Regulation of store-operated and voltage-operated Ca^{2+} channels in the proliferation and death of oligodendrocyte precursor cells by golli proteins

Pablo M Paez[1], Daniel J Fulton, Vilma Spreuer, Vance Handley, Celia W Campagnoni and Anthony T Campagnoni
Semel Institute for Neuroscience and Human Behavior, David Geffen School of Medicine at UCLA, Neuroscience Research Building, 635 Charles Young Drive, Los Angeles, CA 90095, U.S.A.

ABSTRACT

OPCs (oligodendrocyte precursor cells) express golli proteins which, through regulation of Ca^{2+} influx, appear to be important in OPC process extension/retraction and migration. The aim of the present study was to examine further the role of golli in regulating OPC development. The effects of golli ablation and overexpression were examined in primary cultures of OPCs prepared from golli-KO (knockout) and JOE (golli J37-overexpressing) mice. In OPCs lacking golli, or overexpressing golli, differentiation induced by growth factor withdrawal was impaired. Proliferation analysis in the presence of PDGF (platelet-derived growth factor), revealed that golli enhanced the mitogen-stimulated proliferation of OPCs through activation of SOCCs (store-operated Ca^{2+} channels). PDGF treatment induced a biphasic increase in OPC intracellular Ca^{2+}, and golli specifically increased Ca^{2+} influx during the second SOCC-dependent phase that followed the initial release of Ca^{2+} from intracellular stores. This store-operated Ca^{2+} uptake appeared to be essential for cell division, since specific SOCC antagonists completely blocked the effects of PDGF and golli on OPC proliferation. Additionally, in OPCs overexpressing golli, increased cell death was observed after mitogen withdrawal. This phenomenon could be prevented by exposure to VOCC

(voltage-operated Ca^{2+} channel) blockers, indicating that the effect of golli on cell death involved increased Ca^{2+} influx through VOCCs. The results showed a clear effect of golli on OPC development and support a role for golli in modulating multiple Ca^{2+}-regulatory events through VOCCs and SOCCs. Our results also suggest that PDGF engagement of its receptor resulting in OPC proliferation proceeds through activation of SOCCs.

Key words: apoptosis, calcium influx, cell cycle, golli protein, oligodendrocyte, platelet-derived growth factor (PDGF).

INTRODUCTION

The golli–MBP (myelin basic protein) proteins are generated from the *MBP* gene from a promoter that is different from that which controls expression of the classic MBPs. Since their original description (Campagnoni et al., 1993; Pribyl et al., 1993), numerous studies have documented the expression of golli in the immune system and nervous system of several species, including mice and humans. Golli proteins have been found to be up-regulated in adult OPCs (oligodendrocyte precursor cells) and microglia/macrophages around multiple sclerosis lesions (Filipovic et al., 2002). Moscarello et al. (2002)

identified golli as a component of remyelination induced by treatment of demyelinating transgenic mice with paclitaxel (taxol). These studies suggested a dual role of golli proteins in regulating activation of some immune cells as well as the early stages of OPC migration and proliferation.

Ca^{2+} ions are regulators of numerous cellular processes, including survival, motility, apoptosis and differentiation (Berridge et al., 1998), and a growing amount of evidence suggests a critical role for Ca^{2+} in controlling proliferative events (Estacion and Mordan, 1993; Berridge, 1995; Munaron, 2002; Kahl and Means, 2003). The golli proteins are not constituents of myelin, but they appear to play a basic role in the regulation of Ca^{2+} homoeostasis in immune cells and in OPCs (Feng et al., 2006; Paez et al., 2007). Cell membrane depolarization by high potassium (K^+) activates VOCCs (voltage-operated Ca^{2+} channels), while depletion of intracellular Ca^{2+} stores triggers Ca^{2+} influx via SOCCs (store-operated Ca^{2+} channels). Recent work has shown that changes in golli expression alter the magnitude of voltage-sensitive Ca^{2+} currents as well as SOCCs in OPCs, a finding that has potentially important consequences on multiple aspects of OPC maturation and survival (Paez et al., 2007, 2008).

Golli proteins can modulate Ca^{2+} levels during a critical phase of oligodendrocyte development through an action on rates of cell migration and process extension and retraction, presumably post-mitotic events (Paez et al., 2007). We wondered whether golli could also influence earlier events in OPC biology, such as proliferation, through modulation of Ca^{2+} influx. In the present study, we explored the relationship between golli, Ca^{2+} influx and OPC proliferation and survival. We observed that the overexpression of golli promotes OPC proliferation through activation of SOCCs, but at the same time golli increased apoptotic cell death mediated by Ca^{2+} influx through VOCCs. Interestingly, we found that golli were able to modulate the mitogenic effects of PDGF (platelet-derived growth factor), thus providing new insights into the role of golli in the regulation of OPC development.

MATERIALS AND METHODS

Animal experimentation
Animals were maintained by qualified staff in the UCLA Vivarium. The use of these animals was governed by the 'Principles for Use of Animals' and 'Guide for the Care and Use of Laboratory Animals' from the Office for Protection from Research Risks of the National Institutes of Health.

Transgenic mice
Golli-KO (knockout) mouse
We previously generated a golli-KO mouse in which the golli products of the *Mbp* gene were selectively ablated while permitting normal expression of the classic MBPs (Jacobs et al., 2005). Through non-brother–sister crosses, a line was generated that is homozygous for the golli ablation on a background that is 50% 129S7/SvEvBrd and 50% C57BL6/J. A control line (KO control) was established that was also 50% 129S7/SvEvBrd and 50% C57BL6/J, but was negative for the golli ablation. The golli-KO phenotype was observed before keeping the lines separate and then was studied over at least eight generations and remained stable.

JOE (golli J37-overexpressing) mouse
We generated a transgenic mouse that overexpresses the golli J37 isoform in oligodendrocytes under the control of a classic *Mbp* promoter (Martin et al., 2007). In this transgenic mouse, the J37 golli isoform is driven by a 1.9-kb region of the classic *Mbp* promoter, thus directing overexpression of the protein specifically to oligodendrocytes in the CNS (central nervous system) (Reyes et al., 2003). These mice are called JOE mice for golli J37-overexpressing. A line was generated that is heterozygous for the MBP 1.9-J37 transgene on a background that is 50% BALB/cByJ, 37–50% C57BL/6 and 0–12% C3H/He. A control line (JOE control) was established that was also 50% BALB/cByJ, 37–50% C57BL/6 and 0–12% C3H/He.

Primary cultures of cortical oligodendrocytes
Enriched oligodendrocytes were prepared as described by Amur-Umarjee et al. (1993). First, cerebral hemispheres from 1-day-old mice were mechanically dissociated and were plated on poly-D-lysine-coated flasks in DMEM (Dulbecco's modified Eagle's medium) and Ham's F12 (1:1, v/v) (Invitrogen Life Technologies), containing 100 μg/ml gentamycin and supplemented with 4 mg/ml dextrose anhydrous, 3.75 mg/ml Hepes buffer, 2.4 mg/ml sodium bicarbonate and 10% (v/v) FBS (fetal bovine serum) (Omega Scientific). After 24 h, the medium was changed, and the cells were grown in DMEM/Ham's F12 supplemented with 5 μg/ml insulin, 50 μg/ml human transferrin, 30 nM sodium selenite, 10 mM D-biotin, 0.1% BSA (Sigma–Aldrich), 1% (v/v) horse serum and 1% (v/v) FBS. After 9 days, OPCs were purified from the mixed glial culture by the differential shaking and adhesion procedure of Suzumura et al. (1984) and were allowed to grow on poly-D-lysine-coated coverslips in defined culture medium (Agresti et al., 1996) plus 10 ng/ml PDGF-AA and 10 ng/ml bFGF (basic fibroblast growth factor) (Peprotech). OPCs were kept in mitogens (PDGF and bFGF) for 2 days and then induced to exit from the cell cycle and differentiate by switching the cells to a mitogen-free medium (mN2) (Oh et al., 2003). mN2 is DMEM/Ham's F12 supplemented with 4.5 g/l D-glucose, 5 μg/ml insulin, 50 μg/ml human transferrin, 30 nM sodium selenite, 15 nM T_3 (tri-iodothyronine), 10 mM D-biotin, 10 nM hydrocortisone, 0.1% BSA, 1% (v/v) horse serum and 1% (v/v) FBS.

Immunocytochemistry

Cells were stained with antibodies against NG2 (nerve/glial antigen 2), O4 antigen, GC (galactocerebroside), MBP and phospho-histone H3 (Ser[10]) and examined by confocal microscopy. For MBP and phospho-histone H3 (Ser[10]) immunostaining, the cells were rinsed briefly in PBS and fixed in 4% (w/v) buffered paraformaldehyde (pH 7.6) for 30 min at room temperature (20°C). After rinsing in PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature and then processed for immunocytochemistry following the protocol as outlined by Reyes and Campagnoni (2002). Essentially, fixed cells were incubated in a blocking solution (5% goat serum in PBS) followed by an overnight incubation at 4°C with a polyclonal antibody against MBP (1:700) or phospho-histone H3 (Ser[10]) (1:100; Upstate Biotechnology). Staining with anti-NG2 (1:100; Chemicon International), anti-(O4 antigen) (1:20) and anti-GC (1:20) was performed on live cells without permeabilization for 1 h at room temperature before fixation. Cells were then incubated with the appropriate secondary antibodies (1:200; Jackson ImmunoResearch Laboratories), mounted on to slides with Aquamount (Lerner Laboratories), and fluorescent images were obtained using a Olympus spinning-disc confocal microscope. Nuclei were stained with the fluorescent dye Hoechst 33342 (5 µg/ml in 1% DMSO) in order to determine the total number of cells. Quantitative analysis of the results was carried out by counting the antigen-positive and Hoechst 33342-positive cells in 20 randomly selected fields, which resulted in counts of >2000 cells for each experimental condition. Counts of antigen-positive cells were normalized to the counts of total Hoechst 33342-positive cells for each condition.

Cell morphology assessment

Process formation of oligodendrocytes was evaluated by estimating the percentage of O4-positive cells having processes with a length which was equal to or longer than four times the cell body diameter. This method has been used previously for monitoring morphological changes in oligodendrocytes as well as other cell types (Yong et al., 1988, 1991). To obtain the percentage of cells with long processes, fields were randomly chosen on oligodendrocyte-bearing coverslips and all of the O4-positive cells were determined (~500 cells for each experimental condition), as well as the number of oligodendrocytes having processes four times the cell body diameter, as described by Yong et al. (1988, 1991).

Incorporation of BrdU (bromodeoxyuridine)

Pulses of 10 µM BrdU (BD Pharmingen) were applied for 24 h at 0, 24, 48 and 72 h. After each BrdU pulse, cells were fixed and immunostained in order to determine the number of positive cells. The cells were fixed in 4% (w/v) paraformaldehyde in PBS. After treatment with 6 M HCl and 1% (v/v) Triton X-100 to denature nuclear DNA, the cells were incubated in 0.1 M sodium borate (in PBS and 1% Triton X-100) for 10 min. Immunocytochemistry was carried out using an anti-BrdU antibody (1:1000; BD Pharmingen) and an anti-NG2 antibody (1:100; Chemicon International) with the corresponding fluorescent secondary antibodies. The percentage of BrdU-positive cells was estimated on the basis of the total number of NG2-positive cells.

T_c (cell-cycle time) analysis

To examine T_c, mixed glial culture of GFP (green fluorescent protein)-labelled OPCs were used. These experiments were performed using a double-transgenic mouse created by breeding the golli-KO and JOE mice with a line expressing GFP under the control of the *Plp* (proteolipid protein) promoter (Mallon et al., 2002). Mixed glial cultures were prepared as described above (in the Primary cultures of cortical oligodendrocytes section). These cultures were incubated in a stage-top chamber with 5% CO_2 at 37°C, which was placed on the stage of a spinning-disc inverted confocal microscope equipped with a motorized stage, an atmosphere regulator and shutter control. Fluorescent field images were obtained with a specific GFP filter at 6 min intervals. Individual clones of GFP-labelled OPCs (OPC~GFPs) were monitored for a period of 48 h beginning at 2, 4 and 6 DIV (days *in vitro*) before the shake-off. In these time-lapse experiments, ~30 clones were analysed per experimental condition.

SlideBook[TM] 4.1 (Intelligent Imaging Innovations) was used in the analysis of videomicroscopic image sequences. The SlideBook[TM] software allows an investigator to cycle back and forth through the movie files frame-by-frame (minute-by-minute), facilitating the accurate determination of event time. To quantitatively analyse the dynamics of cell division, 120 cytokinetic events were randomly selected from movies at 2, 4 and 6 DIV. Cell proliferation was assessed by calculating the average T_c (time between birth cytokinesis and division cytokinesis) in different OPC~GFP clones. Tracking of cells was performed by visual observation of image sequences as described above. In some cases, the cells were semi-automatically followed in SlideBook[TM] by attaching a number to the cell, which was propagated from frame to frame. Tracking was performed forwards and backwards in time from each cytokinesis event identified to maximize the number of linearly related cytokineses identified.

Cell viability analysis

A Trypan Blue exclusion assay was used to determine cell viability. Following different experimental treatments, OPCs grown on coverslips for 1, 2, 3 and 4 DIV were incubated in medium containing Trypan Blue (0.04% final concentration) for 10 min at 37°C. Viable cells were counted in a total of six experiments, four random fields, on triplicate coverslips.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay

The MTT survival assay was performed as described by Mosmann (1983). The sterile solution of MTT (Molecular Probes) was added to all wells, and the microplate was incubated at 37°C for 45 min. The reaction was stopped by addition of SDS, and the product was quantified by spectrophotometry at 570 nm.

Caspase 3 assay

NucView[TM] 488 caspase 3 substrate (Biotium), a novel cell membrane-permeable fluorigenic caspase substrate designed for detecting caspase 3 activity within live cells in real time, was used in accordance with the manufacturer's recommendations. Briefly, OPC primary cultures were incubated in medium containing NucView[TM] 488 caspase 3 substrate (5 μM final concentration) in a stage-top chamber with 5% CO_2 at 37°C, which was placed on the stage of a spinning-disc inverted confocal microscope. Fluorescent field images were obtained with a specific GFP filter at 6 min intervals for a total of 12 h. SlideBook[TM] 4.1 software was used to assess apoptotic cell death by calculating the percentage of caspase 3-positive cells in a total of five experiments on four random fields.

Calcium imaging

Methods were similar to those described previously (Colwell, 2000; Michel et al., 2002; Paz Soldán et al., 2003). Briefly, a cooled CCD (charge-coupled device) camera (Hamamatsu ORCA-ER) was added to the Olympus spinning-disc confocal microscope to measure fluorescence. To load the dye into cells, the coverslips were washed in serum and Phenol Red-free DMEM, and the cells were incubated for 45 min at 37°C in 5% CO_2 in the same medium containing a final concentration of 4 μM fura 2/AM (fura 2 acetoxymethyl ester) (TefLabs) plus 0.08% Pluronic® F127 (Molecular Probes), then washed four times in DMEM and stored in DMEM for up to 1 h before being imaged (Paz Soldán et al., 2003). Measurements of resting Ca^{2+} levels were made in serum-free HBSS (Hanks balanced salt solution) containing 2 mM Ca^{2+}, but no Mg^{2+}. Other measurements were made in HBSS. Ca^{2+} influx and resting Ca^{2+} levels were measured on individual cells, and the results were pooled from five separate coverslips representing five separate cells preparations for each condition. The fluorescence of fura 2 was excited alternately at wavelengths of 340 and 380 nm by means of a high-speed wavelength-switching device (Lambda DG-4; Sutter Instruments). SlideBook[TM] software was then used to select several 'regions of interest' within the field from which measurements were taken. To minimize bleaching, the intensity of excitation light and sampling frequency was kept as low as possible. In these experiments, measurements were normally made once every 2 s.

Calibration of Ca^{2+} signals

Free [Ca^{2+}] was estimated from the ratio (R) of fluorescence at 340 and 380 nm, using the following equation: [Ca^{2+}] $= K_d \times$ slope factor $\times (R - R_{min})/(R_{max} - R)$ (Grynkiewicz et al., 1985). The K_d was assumed to be 140 nM, whereas values for R_{min} and R_{max} were all determined via calibration methods. An in vitro method (fura 2 Ca^{2+} imaging calibration kit, Molecular Probes) was used to make estimated values. Using this method, glass coverslips were filled with a high-Ca^{2+} (fura 2 plus 10 mM Ca^{2+}), a low-Ca^{2+} (fura 2 plus 10 mM EGTA) or a control solution without fura 2. Each solution also contained a dilute suspension of 15 μm polystyrene microspheres to ensure uniform coverslip/slide separation and facilitate microscope focusing. The fluorescence (F) at 380 nm excitation of the low-Ca^{2+} solution was imaged, and the exposure of the camera was adjusted to maximize the signal. These camera settings were then fixed, and measurements were made with 380 and 340 nm excitation of the three solutions. $R_{min} = F_{340}$ in low-Ca^{2+}/F_{380} in low-Ca^{2+}; $R_{max} = F_{340}$ in high-Ca^{2+}/F_{380} in high-Ca^{2+}; $Sf = F_{380}$ in low-Ca^{2+}/F_{380} in high-Ca^{2+}.

Statistical analysis

Data are presented as means $\pm$ S.E.M. unless noted otherwise. For fura 2 and T_c experiments, statistical comparison between different experimental groups was performed using ANCOVA (analysis of covariance). Measurements of the percentage of positive cells (NG2, O4, GC, MBP, phospho-histone H3, BrdU, Trypan Blue and caspase 3) were carried out using Student's paired t test, in which $P < 0.05$ was defined as statistically significant.

RESULTS

Golli stimulates OPC proliferation in the presence of mitogens

Immediately after shake-off from mixed glial cultures, OPCs were grown in the presence of PDGF-AA (10 ng/ml) and bFGF (10 ng/ml) for 48 h and then induced to exit the cell cycle and to differentiate by transferring the cells to a mitogen-free medium (mN2) (Figure 1A) (Oh et al., 2003). To determine the effect of golli on OPC proliferation, we calculated the percentage of proliferating cells each day over a period of 4 DIV. Since actively proliferating cells duplicate their DNA content, we labelled proliferating OPCs from golli-KO and JOE mice with the thymidine analogue BrdU. Pulses of 10 μM BrdU for 24 h were given at 0 h (immediately after shake-off), 24, 48 and 72 h (Figure 1A). After each BrdU pulse, proliferating progenitors were identified by double immunofluorescence for BrdU and NG2, and the relative number of NG2-positive/BrdU-positive cells was quantified in each cell

population (Figure 1B). In all cell populations, proliferation declined after transfer to mitogen-free medium at 3 and 4 DIV, as expected, but during the proliferative period, cell proliferation was lower in the golli-KO populations and higher in the JOE populations than in controls (Figure 1C). For example, after 1 DIV, the average number of proliferating cells in the golli-KO population (27%) was significantly lower than that of the genotype control group (41%, $P<0.01$) (Figure 1C). In similar experiments, the average percentage of proliferating OPCs in JOE cells was almost 30% higher than of the genotype control cells (52 and 37% respectively, $P<0.001$) (Figure 1C). Golli overexpression did not interfere

with the ability of OPCs to exit the cell cycle after mitogen deprivation, since, in JOE cultures, less than 10% of the cells incorporated BrdU at 4 DIV (Figure 1C). Neither did the absence of golli modify the response of progenitor cells to mitogen withdrawal, since the percentage of NG2-positive/ BrdU-positive cells was also quite low, ranging from 10 to 15% at 3 DIV and from 5 to 10% at 4 DIV (Figure 1C). Additionally, we evaluated the total number of NG2-positive cells in golli-overexpressing cultures at 1 and 2 DIV. In agreement with our previous findings, golli overexpression was able to significantly increase the total number of NG2-positive cells by ~40% after 1 DIV (1475 ± 34 cells for JOE,
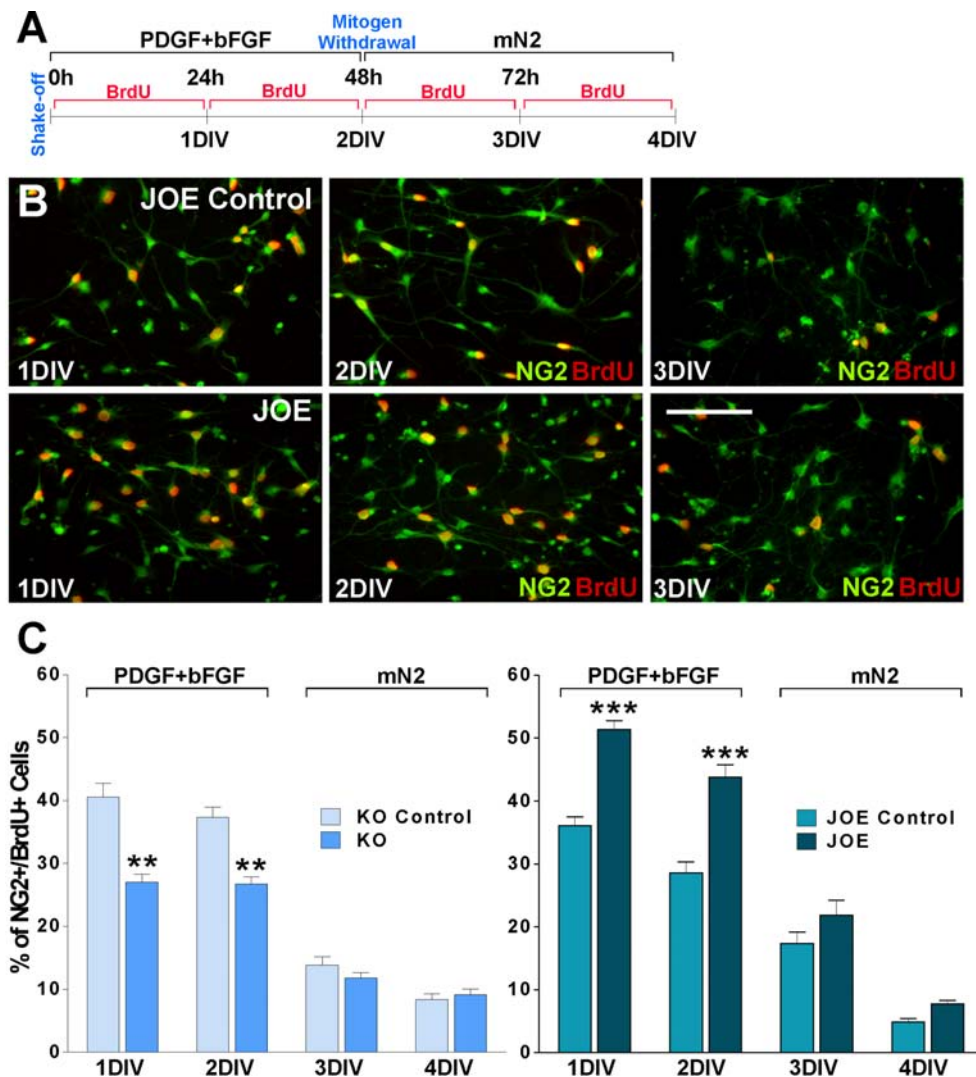

Figure 1   Golli modulates cell poliferation in OPCs
(**A**) Pure OPC cultures from golli-KO and JOE mice were grown for 2 days (i.e. 1 and 2 DIV) in defined culture medium plus PDGF (10 ng/ml) and bFGF (10 ng/ml). Then, the medium was changed, and the cells were cultured in a mitogen-free medium (mN2) for another 2 days (i.e. 3 and 4 DIV). Pulses of 10 μM BrdU for 24 h were begun at 0, 24, 48 and 72 h. After each BrdU pulse, cells were fixed and immunostained with anti-BrdU and anti-NG2 antibodies. (**B**) Photomicrographs showing NG2-positive/BrdU-positive cells at 1, 2 and 3 DIV. Green shows NG2 immunostaining, and red shows BrdU immunostaining. Scale bar, 80 μm. (**C**) The percentage of NG2-positive/BrdU-positive cells under each condition was compared with respective controls. Results are means±S.E.M. for four independent experiments. **$P<0.01$, ***$P<0.001$, compared with control cells.

compared with 1049 ± 52 cells for JOE control, $P<0.01$) and by $\sim 30\%$ after 2 DIV (1132 ± 57 cells for JOE, compared with 789 ± 62 cells for JOE control, $P<0.01$).

Confirmation of the BrdU cell proliferation results in the JOE OPC population was made using an anti-(phospho-histone H3) (Ser[28]) antibody as a marker of mitotic cells. Phosphorylation of histone H3 at Ser[10] and Ser[28] is very important for chromosome condensation and segregation during mitosis (Wei et al., 1999). The phosphorylation of histone H3 at Ser[28] occurs in chromosomes predominantly during early mitosis and coincides with the initiation of mitotic chromosome condensation in various mammalian cell lines (Goto et al., 1999). After 1 and 2 DIV in the presence of mitogens, there were more H3-positive cells in the JOE cell population than in the JOE control cells (Figure 2), but 24 h after mitogen withdrawal (3 DIV), no significant differences between groups were found (Figure 2B).

An increase in JOE OPC divisions and a decrease in golli-KO cell proliferation could be due to a change in cell-cycle kinetics. To explore this possibility, we measured the T_c of the golli-KO, JOE and control cells by performing time-lapse imaging in mixed glial cultures using OPC~GFPs. The OPCs used in these experiments were obtained from double-transgenic mice created by breeding the golli-KO and JOE mice with a line expressing GFP under the control of the *Plp* promoter (Mallon et al., 2002). In these mice, GFP expression provides a convenient marker for OPCs in the imaging experiments in mixed glial cultures. We imaged individual clones of OPC~GFPs in the presence of an astrocyte monolayer, a known source of growth factors such as PDGF, bFGF and IGF-1 (insulin-like growth factor 1) (Hicks and Franklin, 1999). These experiments were performed for a period of 48 h, beginning at 2, 4 and 6 DIV before the shake-off (Figure 3A). We denote days *in vitro* in experiments in the mixed cultures with the lower-case div. In these time-lapse experiments, cell proliferation was assessed by calculating the average T_c in different OPC~GFP clones. T_c was defined as the period between when a cell was first generated by cytokinesis and when that cell subsequently divided, giving birth to two daughter cells. The time at which cytokinesis occurred was considered to be the first appearance of a distinct border between two daughter cells in videomicroscopic image sequence (see the Materials and methods section). Examples of cytokinetic events in OPC~GFPs from golli-KO and JOE mice grown for 4 days on an astrocyte monolayer (4 div) are shown in Figure 3(B). At 2 div, control OPCs showed a T_c of ~ 24 h, whereas the golli-KO cells showed a T_c of ~ 29 h. In contrast, the T_c of JOE OPCs was decreased to ~ 18 h at 2 div and ~ 14 h at 4 div (Figure 3C).

These results indicated by several independent measures that golli promoted OPC proliferation in the presence of growth factors, i.e. an increase in the number of BrdU-positive cells, a decrease in the T_c and an increase in the number of cells in S-phase. This effect disappeared when PDGF and bFGF were removed from the culture medium, suggesting that golli does not influence the ability of OPCs to exit from the cell cycle in response to mitogen deprivation.

After mitogen withdrawal, cells overexpressing golli are more sensitive to cell death

The effect of golli expression on OPC survival was examined using the Trypan Blue exclusion assay and the MTT quantitative colorimetric method for cell viability. The results revealed statistically significant differences in the percentage of Trypan Blue-positive (dead) cells between control and JOE OPCs. At both 3 and 4 DIV, JOE cultures showed a significant increase in the number of Trypan Blue-positive cells (Figure 4A). Similar results were found using the MTT assay
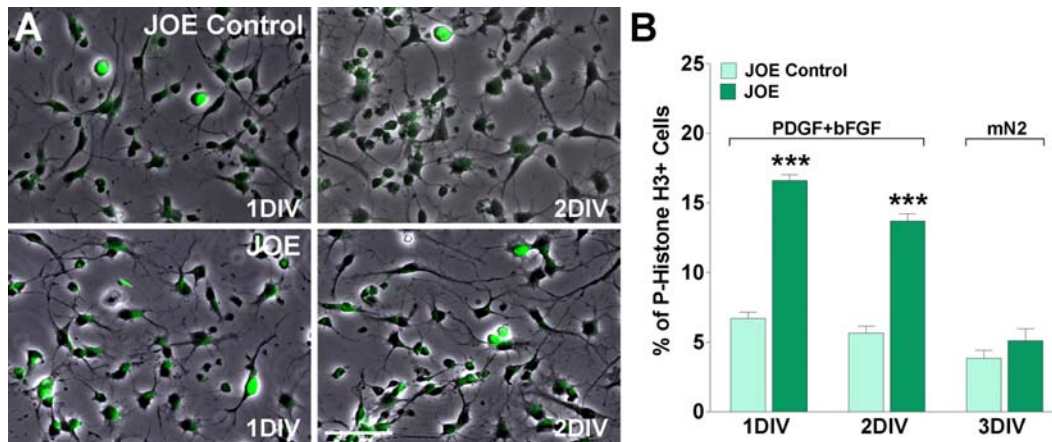

Figure 2   Effects of golli on histone H3 phosphorylation
OPCs from JOE mouse were cultured as indicated in Figure 1(A). (**A**) Immunofluorescent images of JOE cells immunostained with anti-(phospho-histone H3) antibody superimposed upon brightfield at 1 and 2 DIV. Scale bar, 40 μm. (**B**) The percentage of phospho-histone H3-positive cells in each experimental condition was measured as described in the Materials and methods section. Results are means $\pm$ S.E.M. for four independent experiments. ***$P<0.001$ compared with control cells.
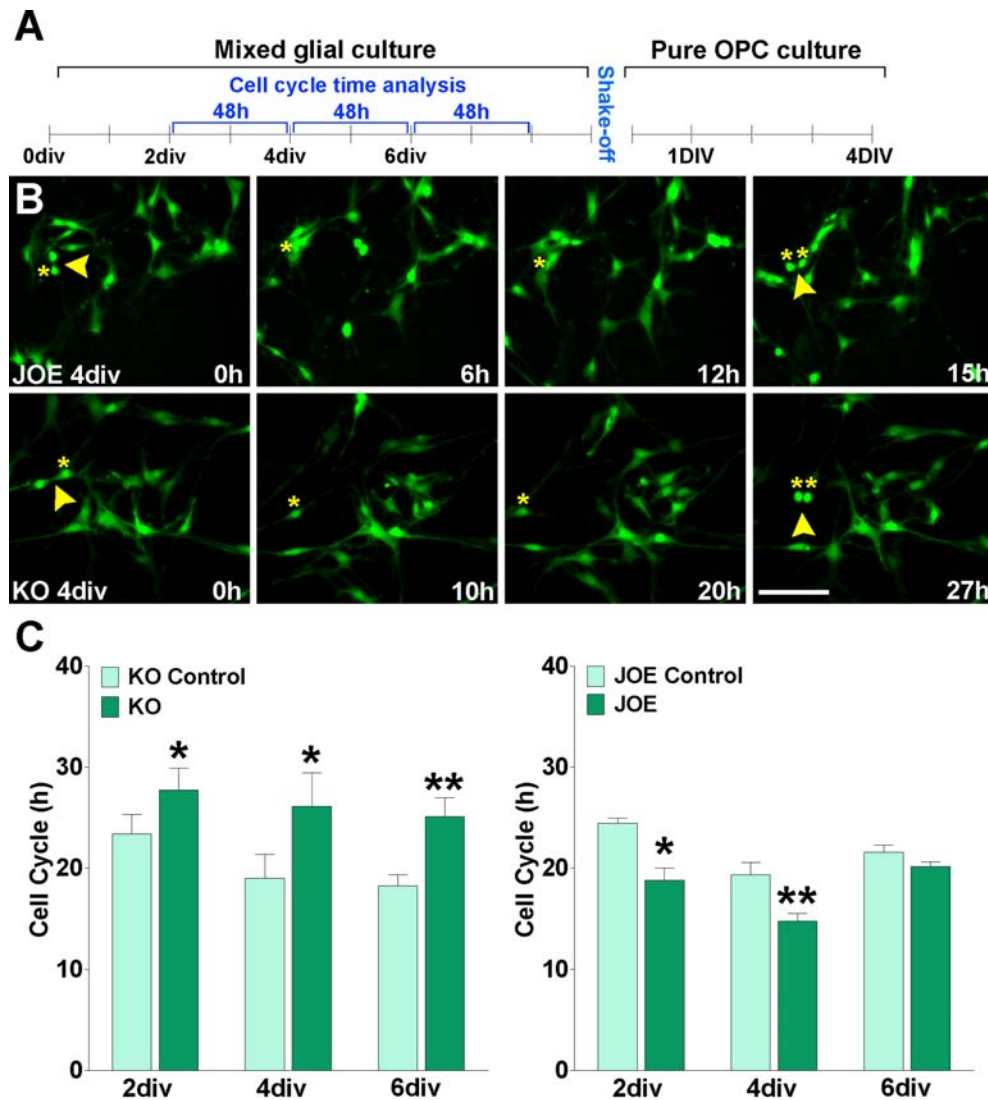
Figure 3   Effect of golli on cell-cycle length
(**A**) Mixed glial cultures were incubated in a stage-top chamber with 5% CO_2 at 37°C, which was placed on the stage of a spinning-disc inverted confocal microscope. OPC~GFP clones were imaged with a specific GFP filter at 6 min intervals for a period of 48 h beginning at 2, 4 and 6 div before the shake-off. (**B**) Time-lapse series of OPC~GFP clones from golli-KO and JOE mice grown for 4 days on an astrocyte monolayer (4 div). Yellow arrowheads designate cytokinesis events. Tracking of cells between birth cytokinesis and division cytokinesis was performed by attaching a yellow asterisk to the cell, which was propagated from frame to frame. Each frame represents a single section of a time-lapse video sequence taken at the time indicated. Scale bar, 60 μm. (**C**) Estimated T_c values were determined as described in the Materials and methods section for each experimental condition. Results are means $\pm$ S.E.M. for four independent experiments. *$P < 0.05$, **$P < 0.01$ compared with control cells.

for live cells: after 3 DIV, OPC viability was higher in control cells than in the JOE group (Figure 4B). In contrast, golli-KO OPCs showed no significant changes in cell viability relative to control using either the Trypan Blue exclusion assay or the MTT technique (Figure 4), suggesting that the decrease in BrdU incorporation in the golli-KO cells is not a consequence of cell death.

We also examined the role of golli on OPC apoptotic cell death in golli-KO and JOE OPCs after 3 DIV. Sequential activation of caspases plays a central role in the execution

phase of cell apoptosis. Members of the caspase family of proteases have been identified as crucial mediators of the complex biochemical events associated with apoptosis. Caspase 3 is a key effector in the apoptotic pathway, amplifying the signal from initiator caspases and signifying full commitment to cellular disassembly. For these studies, we used a real-time caspase 3 assay, which detects caspase 3 activity within individual living cells. This assay is bifunctional in that it is able to detect both intracellular caspase 3 activity and also stain the cell nucleus, which undergoes morpho-
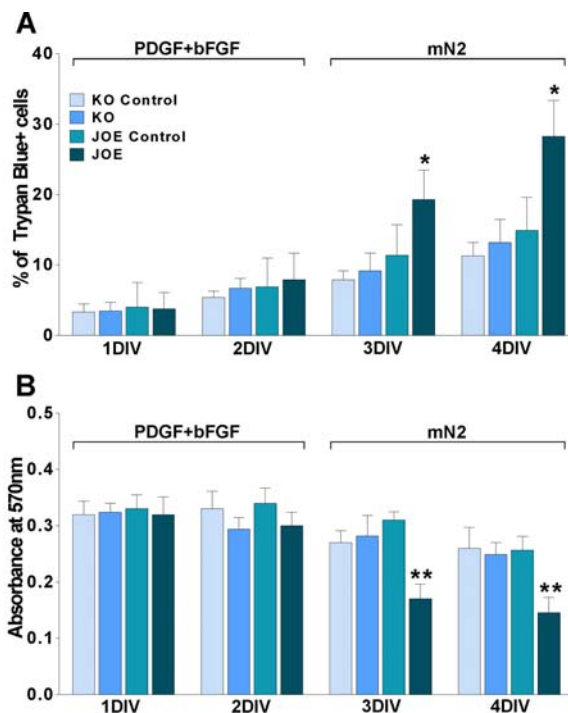
Figure 4 Effect of golli on cell survival
OPCs were cultured as indicated in Figure 1(A). (**A**) The percentage of Trypan Blue-positive cells in each genotype was determined as described in the Materials and methods section. (**B**) Golli-KO and JOE cell viability was evaluated using the MTT survival assay. Results are means ± S.E.M. for two independent experiments, with duplicate cultures for each experimental condition. *$P<0.05$, **$P<0.01$ compared with control cells.

logical changes during the apoptotic process. Examples of such measurements are shown in Figure 5(A). Consistent with the MTT and Trypan Blue results, the combined use of real-time confocal microscopy and the caspase 3 indicator revealed that JOE OPCs at 3 DIV displayed a significant increase in the percentage of caspase 3-positive cells after 12 h (Figure 5B). Parallel experiments with golli-KO compared with control OPCs showed no increase in caspase labelling (Figure 5B).

Golli expression plays a role in OPC differentiation

There is an obligate relationship between proliferation and differentiation, because cell-cycle exit is a prerequisite for oligodendrocyte differentiation (Casaccia-Bonnefil and Liu, 2003). The results described above clearly indicate that endogenous golli expression modulates OPC proliferation and survival. Since these two events are essential for OPC lineage progression, we investigated further the effect of golli on OPC differentiation. Primary cultures of OPCs were kept in mitogens (PDGF and bFGF) for 2 DIV and then induced to exit the cell cycle by switching the cells to a mitogen-free medium (mN2). Under these conditions, there is a general

decline in early immunocytochemical markers, such as NG2, and an increase in intermediate (e.g. O4) and mature (e.g. GC, MBP) markers consistent with differentiation of the OPCs (Figure 6). Analysis of these markers showed that, in cells lacking golli, the number of cells positive for the most mature oligodendrocyte markers, GC and MBP, was significantly lower than in their corresponding genotype controls (Figures 6C and 6D). OPCs from both control and golli-KO mice progressively acquired O4, GC and MBP immunoreactivity, but the percentage of golli-KO cells positive for these antigens was always lower than that found in control cells. After removal of growth factors (3 and 4 DIV), the number of cells positive for O4, GC and MBP increased by approx. 50% in control cultures, whereas in the golli-KO OPCs, there was still no significant change in the number of GC-positive or MBP-positive cells over this period, suggesting a lag in the differentiation of OPCs in the absence of golli after mitogen withdrawal. Consistent with this, we observed a significantly greater decrease in the percentage of immature NG2-positive cells in control compared with golli-KO OPCs (Figure 6A). As shown in Figure 7, similar results were obtained in JOE OPCs. After 1 DIV, the number of cells expressing the intermediate marker O4 increased significantly, and there was a steady decline in the number of cells expressing the immature marker NG2 in the control and JOE cells populations (Figures 7A and 7B). However, after removing the growth factors from the culture medium, the increase in the number of O4-, GC- and MBP-positive cells was only seen in control cells (Figures 7B, 7C and 7D). These results suggested that differentiation of OPCs was sensitive to the levels of golli expressed in the cells.

As expected, in all genotypes, the cells were mostly bipolar during the first 2 DIV in the presence of PDGF and bFGF. However, removal of growth factors increased the morphological differentiation of the cells towards a more complex cell shape similar to that observed in post-mitotic more mature oligodendrocytes. As described by Jacobs et al. (2005), analysis of the morphological complexity of oligodendrocytes (Sperber and McMorris, 2001) showed that, although the golli-KO oligodendrocytes elaborated processes and membrane sheets, these were not as extensive as those elaborated by control oligodendrocytes (Jacobs et al., 2005). In contrast, JOE OPCs were generally flattened and were roughly bipolar during the first 2 DIV, but after growth factor withdrawal at 3 and 4 DIV, they elaborated larger processes and membrane sheets (Figure 8A). The observed morphological differences were confirmed by quantitative assessment of the percentage of OPCs with processes having lengths equal to or greater than four times the mean cell body diameter of the OPC population (Figure 8B). During the first 2 DIV, JOE cells meeting this criterion comprised approx. 20% of the population. But, after these cells were transferred to differentiation medium without growth factors, the proportion of JOE cells with large processes rose to ~60% (Figure 8B).
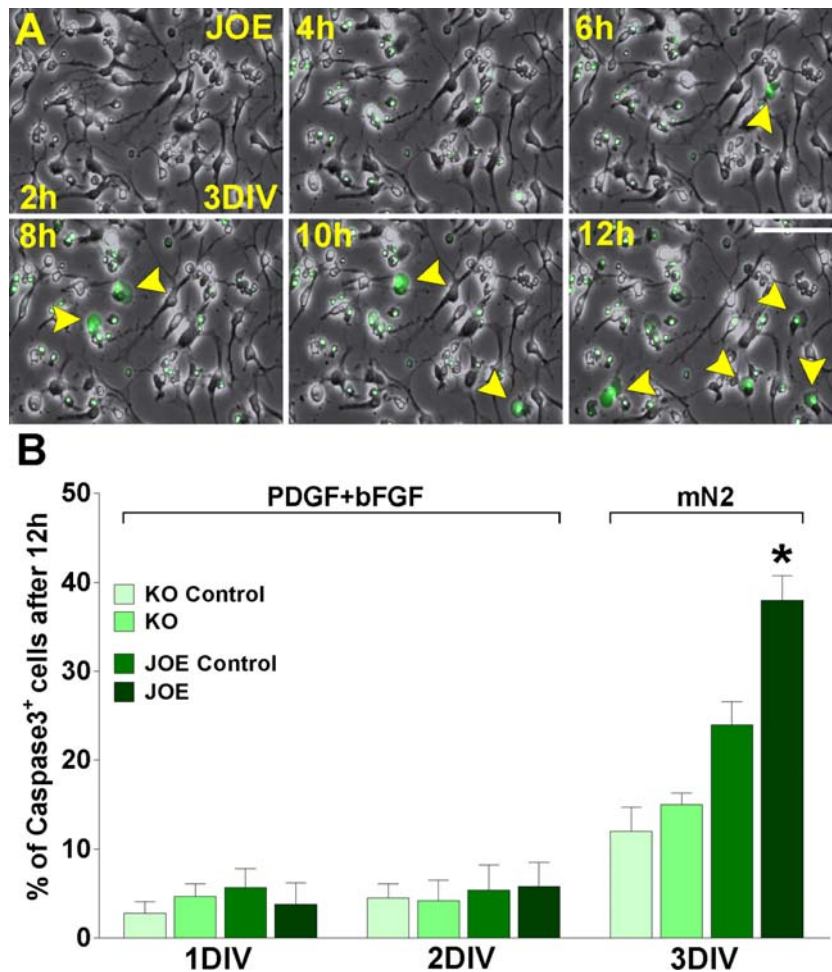
Figure 5   Golli overexpression can lead to increased death of OPCs
OPCs were cultured as indicated in Figure 1(A). (**A**) Real-time caspase 3 assay, using NucView™ 488 caspase 3 substrate, was performed as described in the Materials and methods section. Fluorescent field images were obtained with a specific GFP filter at 6 min intervals for a period of 12 h beginning at 3 DIV. Brightfield images were superimposed to show the cell morphology. Yellow arrowheads designate some apoptotic JOE OPCs (caspase 3-positive cells). Images shown were taken at 2 h intervals. Scale bar, 60 μm. (**B**) Golli-KO and JOE cell death was evaluated by measuring the percentage of caspase 3-positive cells for a period of 12 h at 1, 2 and 3 DIV. Results are means ± S.E.M. for five independent experiments. *$P < 0.05$ compared with control cells.

These results are consistent with the notion that golli promotes process/membrane sheet formation, taking OPCs to an apparently more mature morphological state. However, the available data suggest that golli does not affect the genetic programme leading to the establishment of the composition of myelin. These results are consistent with previous studies on golli overexpression in transfected oligodendrocyte cell lines (Reyes and Campagnoni, 2002).

Golli increases the response of OPCs to PDGF

PDGF is the most potent proliferative factor for OPCs, and the effect of golli on OPC proliferation occurs only in the presence of growth factors. Since golli modulation of PDGF mitotic activity could be a possible explanation for our results, we decided to investigate further the effect of golli on PDGF mitogenic action. OPC primary cultures were treated with different concentrations of PDGF, in the presence of bFGF (10 ng/ml), for the first 24 h after plating. In order to examine the ability of PDGF to induce proliferation of control, golli-KO and JOE cells, the relative number of NG2-positive/BrdU-positive OPCs was quantified in each cell population. In Figure 9, the percentage of NG2-positive/BrdU-positive cells in golli-KO and JOE OPCs compared with genotype control OPCs is plotted as a function of PDGF concentration. At all concentrations of growth factor, proliferation of the golli-KO OPC population was significantly lower than controls. The average percentage of proliferating OPCs in the golli-KO population was found to be almost half of the golli-KO control cells at all PDGF concentrations tested (Figure 9). In contrast, proliferation of the JOE OPCs was significantly higher than genotype controls (Figure 9). At
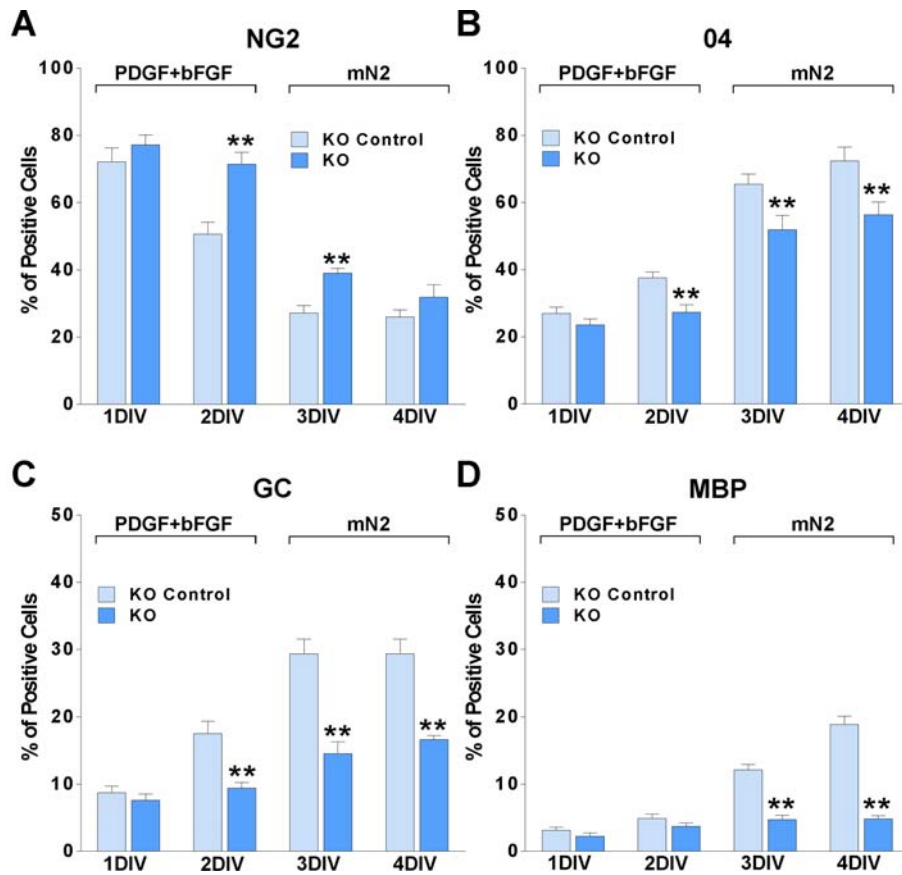
Figure 6   Absence of golli delays OPC differentiation
Pure OPCs from control and golli-KO mice were cultured for 2 days (1 and 2 DIV) in defined culture media plus PDGF (10 ng/ml) and bFGF (10 ng/ml). Then, the medium was changed and the cells were cultured in a mitogen-free medium (mN2) for another 2 days (3 and 4 DIV). OPCs were immunostained with anti-NG2 (**A**), O4 (**B**), anti-GC (**C**) and anti-MBP (**D**) antibodies and the fluorescent dye Hoechst 33342. The percentage of positive cells under each experimental condition was measured as described in the Materials and methods section. Results are means $\pm$ S.E.M. for four independent experiments. **$P < 0.01$ compared with control cells.

higher PDGF concentrations (40–80 ng/ml), the percentage of JOE NG2-positive/BrdU-positive OPCs was almost ~50% higher than control OPCs.

PDGF is the best characterized and the most active proliferative factor for OPCs. It exerts its effect through interaction with receptors bearing intrinsic tyrosine kinase activity (Taniguchi, 1995). Ca^{2+} signals activated after membrane receptor recruitment is one of the most conserved responses triggered in the target cells in which PDGF exert a mitogenic activity (Fatatis and Miller, 1997). It was shown previously that golli proteins play a key role in the regulation of Ca^{2+} homoeostasis in OPCs (Paez et al., 2007). For this reason, we examined the effect of PGDF on intracellular Ca^{2+} levels in control and JOE OPCs. PDGF (40 ng/ml) induced a biphasic increase in OPC intracellular Ca^{2+} ($[Ca^{2+}]_{int}$). The first phase consisted of a sharp peak characteristic of a large transient increase in $[Ca^{2+}]_{int}$ (transient phase) (Figure 10A), which was followed by a second phase of slowly declining internal Ca^{2+} concentrations (sustained phase) (Figure 10A). The amplitude of the first phase of $[Ca^{2+}]_{int}$ increase induced

by PDGF in the JOE cells was not significantly different from those observed in control OPCs (188 ± 3.4 and 191 ± 2.8 nM respectively, $n=45$) (Figures 10A and 10B). However, during the first 2 min of the second (sustained) phase, $[Ca^{2+}]_{int}$ was significantly higher in JOE cells than in control cells (148 ± 4.2 and 116 ± 3.6 nM respectively, $n=45$, $P<0.05$) (Figures 10A and 10B).

After pre-incubation of the cells in the absence of external Ca^{2+} the second (sustained) phase of increased internal Ca^{2+} levels disappeared (Figure 10C), but the first (transient) $[Ca^{2+}]_{int}$ peak was unchanged. In contrast, treatment of the cells with thapsigargin (Tg) in Ca^{2+}-free medium to deplete intracellular stores, suppressed the transient $[Ca^{2+}]_{int}$ increase as well as the sustained phase of higher internal Ca^{2+} levels (Figure 10D). These results indicated that the first (transient) $[Ca^{2+}]_{int}$ increase after PDGF treatment was mediated primarily by Ca^{2+} release from intracellular stores and that the sustained phase was mediated by extracellular Ca^{2+} influx into the cells. To analyse the effect of golli on Ca^{2+} entry induced by PDGF, a protocol was used that involved store
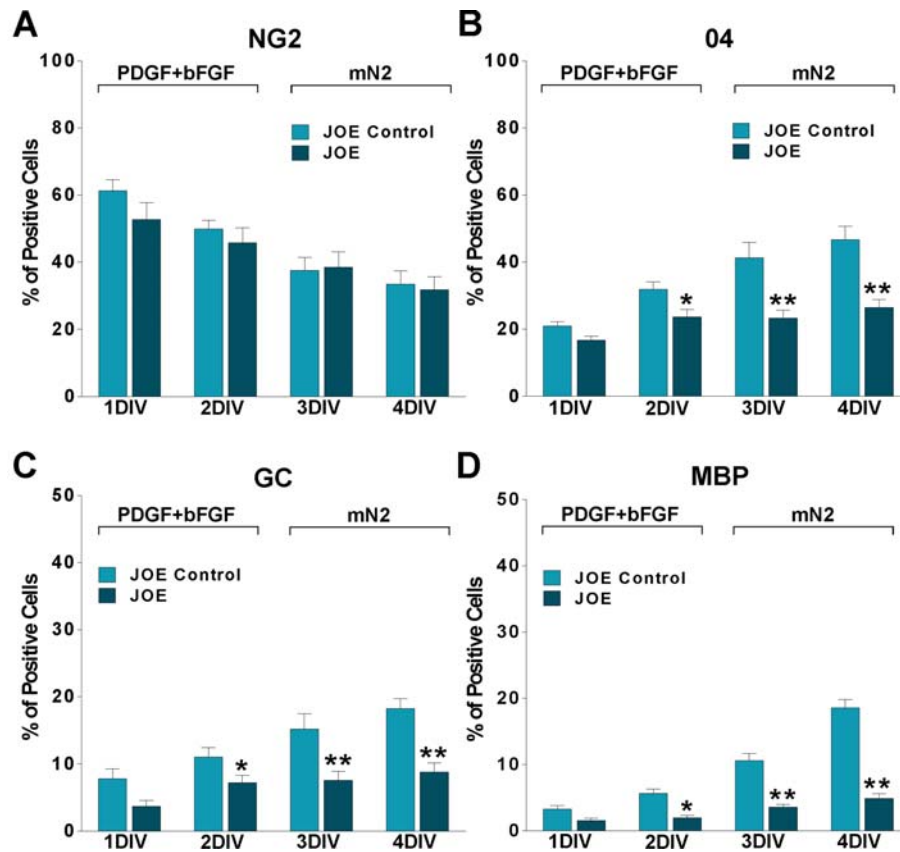
Figure 7   Differentiation of OPCs is sensitive to golli levels in the cells

OPCs from a JOE mouse were cultured as indicated in Figure 6. OPCs were immunostained with anti-NG2 (**A**), anti-O4 (**B**), anti-GC (**C**) or anti-MBP (**D**) antibodies and the fluorescent dye Hoechst 33342. The percentage of positive cells under each experimental condition was measured as described in the Materials and methods section. Results are means ± S.E.M. for four independent experiments. *$P<0.05$, **$P<0.01$ compared with control cells.
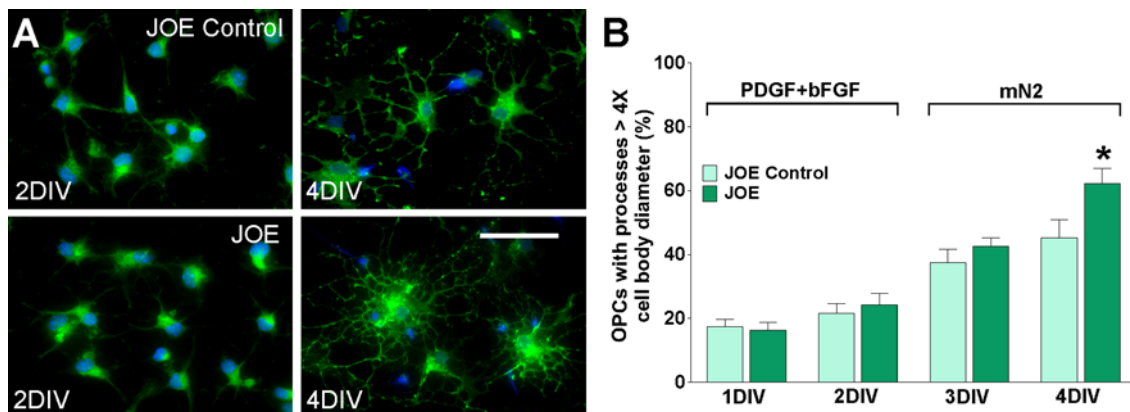

Figure 8   Golli enhances the morphological complexity of OPCs and oligodendrocytes

OPCs were cultured as indicated in Figure 6. (**A**) Fluorescent images of JOE cells immunostained with an anti-O4 antibody at 2 and 4 DIV. Scale bar, 35 μm. (**B**) O4-positive cells were scored for morphological complexity (see the Materials and methods section), and the percentage of cells with processes greater or equal to four times the cell body diameter was plotted. Results are means ± S.E.M. for four independent experiments. *$P<0.05$ compared with control cells.
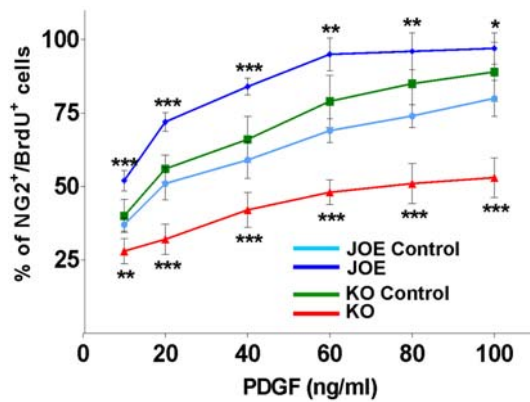
Figure 9 Golli enhances the proliferative effect of PDGF on OPCs
Pure OPC cultures from golli-KO and JOE mice were treated, during the first 24 h after plating (1 DIV), with different concentrations of PDGF in the presence of bFGF (10 ng/ml). To test the mitogenic effect of PDGF, the relative number of NG2-positive/BrdU-positive OPCs was quantified in each cell population. Results are means ± S.E.M. for four independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control cells.

depletion by PDGF application in the absence of extracellular Ca^{2+}, followed by activation of Ca^{2+} entry owing to re-addition of Ca^{2+} to the extracellular medium. As shown in Figure 10(E), application of PDGF to the cells in Ca^{2+}-free medium evoked an initial rise in $[Ca^{2+}]_{int}$ which was due to release from intracellular stores. After this increase in $[Ca^{2+}]_{int}$ had returned to baseline levels, Ca^{2+} was added to the extracellular medium, resulting in an elevation in $[Ca^{2+}]_{int}$ due to influx across the plasma membrane. Re-exposure to Ca^{2+}-containing medium triggered a significantly larger increase in the fura 2 signal in JOE cells in comparison with responses in controls (Figures 10E and 10F). Interestingly, opposite effects were observed in golli-KO OPCs (Figures 10E and 10F).

We then examined the Ca^{2+} channels responsible for the increase in $[Ca^{2+}]_{int}$ during the sustained phase. The transient increases in $[Ca^{2+}]_{int}$ stimulated by PDGF, in both control and JOE cells, and the Ca^{2+} sustained phase were unaffected by pre-treatment with 50 μM verapamil, a VOCC blocker (Figure 11A). However, in both control and JOE cells, 25 μM 2-APB (2-aminoethoxydiphenyl borate), a specific SOCC inhibitor, completely abolished the sustained phase in the $[Ca^{2+}]_{int}$ increase due to PDGF treatment (Figure 11B). This was also manifest as a decrease in both the peak Ca^{2+} signal and integrated Ca^{2+} transient following Ca^{2+} re-addition (Figures 11C and 11D). MRS-1845, another specific blocker of store-operated Ca^{2+} entry, exhibited a similar degree of inhibition upon Ca^{2+} influx following store depletion with PDGF (Figures 11C and 11D). Thus the first phase of $[Ca^{2+}]_{int}$ increase in OPCs after PDGF treatment was due to Ca^{2+} release from intracellular Ca^{2+} stores, whereas the second (sustained) phase of $[Ca^{2+}]_{int}$ increase was due to extracellular Ca^{2+} influx through SOCCs located on the plasma membrane.

These results indicate that golli may influence the effects of PDGF on OPCs by modulating Ca^{2+} influx through SOCCs. Further evidence for this interpretation came from exposure of the cells to BrdU for 24 h in the presence of channel blockers to measure proliferation (Figure 11E). These experiments revealed that pharmacological blockers of SOCCs inhibited the golli-enhanced proliferation of OPCs in the presence of PDGF, whereas there was no effect in the presence of the VOCC blocker verapamil. Additionally, these experiments are the first to demonstrate that extracellular Ca^{2+} uptake through SOCCs is an important component in the mechanism of PDGF on OPC proliferation.

High VOCC activity induces apoptosis in JOE OPCs

In a second set of experiments, we explored the hypothesis that the effect of golli on cell death could also be a consequence of golli-dependent Ca^{2+} homoeostasis in OPCs. We performed live imaging experiments to examine and correlate cell death with Ca^{2+} changes in OPC cultures. Addition of 20 mM K^+ to the bathing medium, a manipulation that affects $[Ca^{2+}]_{int}$ by depolarizing the membrane potential and activating VOCCs, caused an increase of the percentage of caspase 3-positive cells (Figure 12A). These effects were more marked in JOE OPCs (Figure 12A). A reduced effect compared with controls was noted in golli-KO OPCs, which appeared to be more resistant to apoptosis than control cells after high K^+ treatment (Figure 12B). Importantly, differences in the percentage of caspase 3-positive cells between genotypes were blocked by verapamil and were abolished in the absence of Ca^{2+} (Figures 12A and 12B), confirming that Ca^{2+} influx via VOCCs are directly involved in golli-mediated modulation of OPC death. Similar results were found using the MTT assay for live cells. After 3 DIV, as shown in Figure 4(B), OPC viability was higher in control cells than in the JOE group, but here the differences in cell viability were abolished by the VOCC inhibitors verapamil and Cd^{2+}, and the absence of extracellular Ca^{2+} (Figure 12C).

DISCUSSION

Oligodendrocytes and their functions are impaired by the selective ablation of golli proteins due to abnormalities in Ca^{2+} uptake and hypomyelination (Jacobs et al., 2005). Similarly, targeted overexpression of golli in oligodendrocytes also impairs Ca^{2+} homoeostasis and myelination *in vivo* and *in vitro* (Martin et al., 2007; Paez et al., 2007). Consequently, OPCs isolated from golli-KO and JOE mice provide useful reagents for probing regulation of Ca^{2+} channels by the golli proteins as well as the role of Ca^{2+} channels in OPC function.
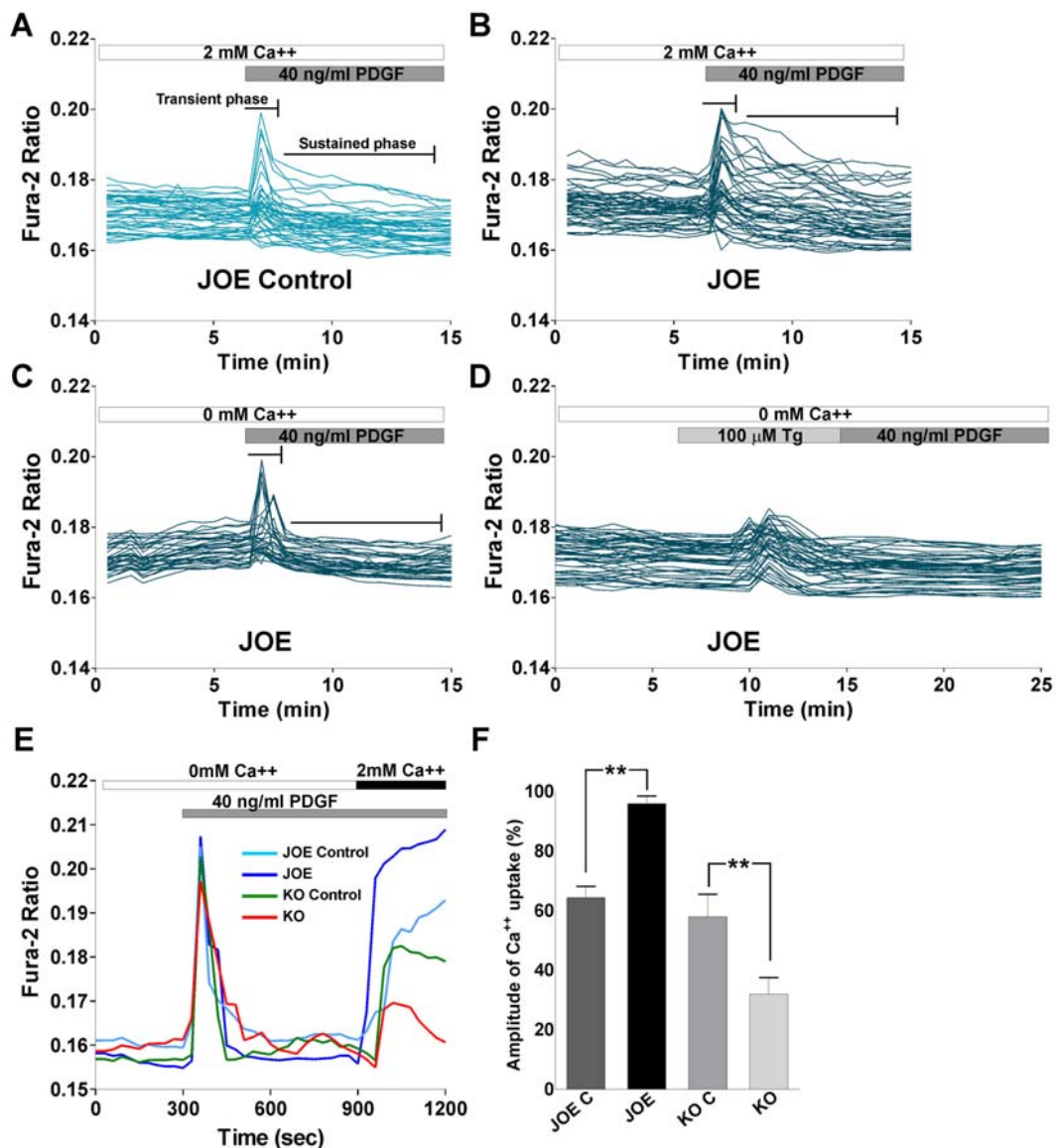
Figure 10 **Effect of golli on internal biphasic Ca^{2+} response to PDGF**
(**A** and **B**) Fura 2 ratio of Ca^{2+} response to 40 ng/ml PDGF in OPCs from control and JOE mice at 1 DIV. (**C**) JOE OPCs treated with 40 ng/ml PDGF in the absence of extracellular Ca^{2+}. (**D**) JOE OPCs were pre-treated with 100 μM thapsigargin (Tg) in Ca^{2+}-free medium to deplete intracellular stores, and then treated with 40 ng/ml PDGF. Note that each trace corresponds to a single OPC. (**E**) Averaged Ca^{2+} response to PDGF stimulation in golli-KO and JOE OPCs at 1 DIV ($n>500$ cells for each genotype). Following depletion of the intracellular Ca^{2+} store by PDGF treatment in the absence of extracellular Ca^{2+}, 2 mM Ca^{2+} was added back to the culture medium. The times of addition of PDGF and Ca^{2+}-containing external solutions are indicated by the horizontal bars. (**F**) Averaged data (means $\pm$ S.E.M., $n>500$ cells for each genotype) indicating the integrated Ca^{2+} signal (i.e. area under the Ca^{2+} trace) measured for 5 min following re-addition of extracellular Ca^{2+}. **$P<0.01$ compared with control cells (C).

OPC proliferation is modulated by golli

Proliferation of OPCs is clearly important for normal myelination as well as for remyelination in demyelinating diseases. The present study indicates that golli overexpression increases the proliferation rate of OPCs as assessed by several parameters including a decrease in the T_c. In contrast, golli-KO OPCs proliferated less robustly and showed a significant increase in the length of the cell cycle. Thus it is likely that

golli promotes enhanced proliferation of OPCs in the presence of mitogens. In this regard, Filipovic and Zecevic (2005) reported that LPS (lipopolysaccharide)-induced inflammation promoted OPC proliferation through secretion of golli proteins from activated microglia.

The differences we noted in cell proliferation in the two genotypes are likely to be explained by changes in the cell cycle duration, since the length of T_c could progressively
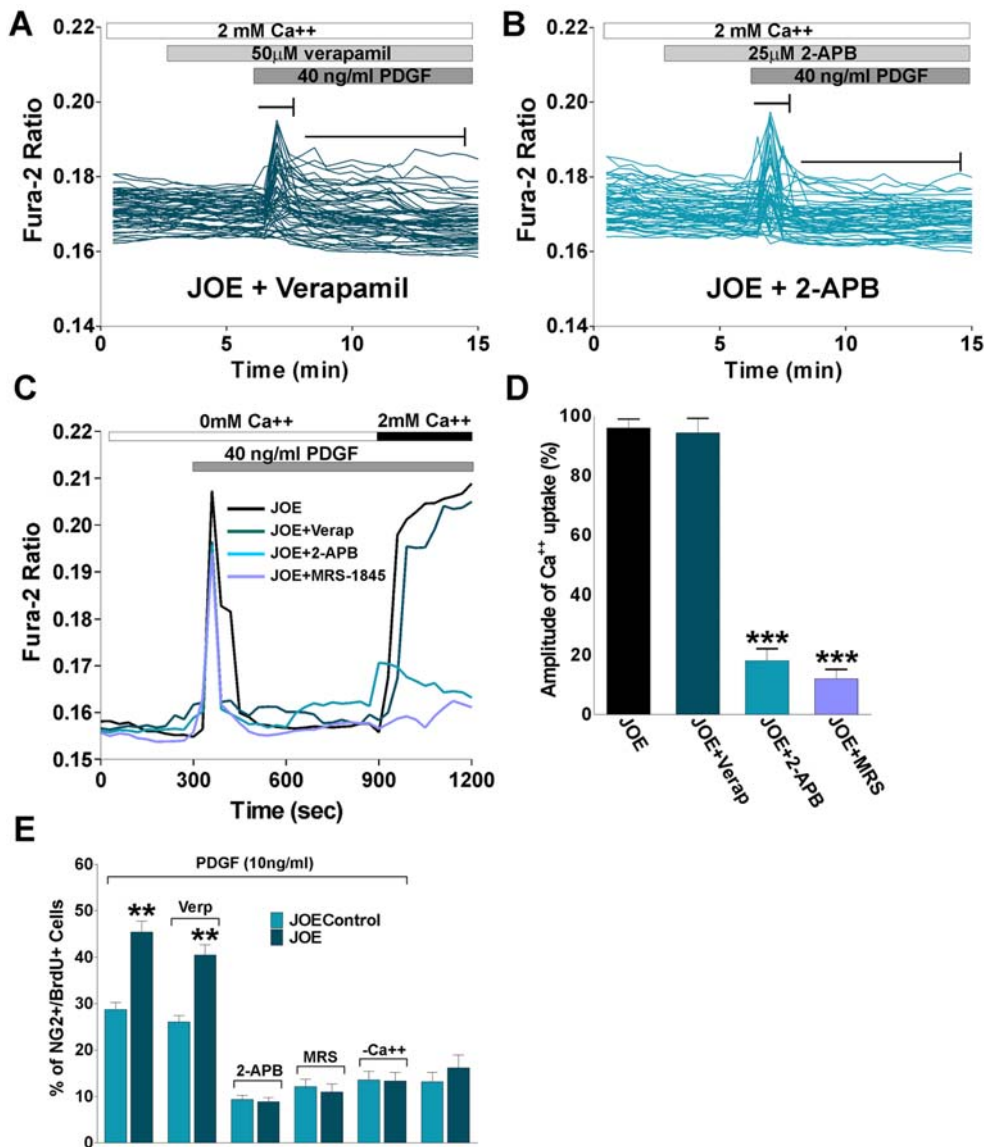
Figure 11   **The first phase of the PDGF–induced increase is internal Ca^{2+} is due to Ca^{2+} release from stores and the second phase is due to Ca^{2+} uptake through SOCCs**

(**A** and **B**) JOE cells were treated with verapamil (50 µM) or 2-APB (25 µM) and then stimulated with PDGF (40 ng/ml). Note that each trace corresponds to a single OPC and the times of addition of verapamil, 2-APB and PDGF are indicated by the horizontal bars. (**C**) Averaged Ca^{2+} response to PDGF stimulation in JOE OPCs (n>500 cells). Following depletion of the intracellular Ca^{2+} store by PDGF, extracellular Ca^{2+} (2 mM) was added back to the cells in the presence of verapamil (Verap; 50 µM), 2-APB (25 µM) or MRS-1845 (20 µM). The times of addition of PDGF and Ca^{2+}-containing external solutions are indicated by the horizontal bars. (**D**) Averaged data (means ± S.E.M., n>500 cells for each experimental condition) indicating the integrated Ca^{2+} signal (i.e. area under the Ca^{2+} trace) measured for 5 min following re-addition of extracellular Ca^{2+}. ***P<0.001 compared with JOE cells. (**E**) Pulses of 10 µM BrdU for 24 h were begun at 24 h after the shake-off. BrdU incorporation was determined in JOE OPCs stimulated with PDGF (10 ng/ml) in the presence of verapamil (Verp; 50 µM), 2-APB (25 µM) or MRS-1845 (MRS; 20 µM) or in the absence of extracellular Ca^{2+} (-Ca^{++}). Results are means ± S.E.M. for five independent experiments. **P<0.01 compared with control cells.

increase the probability that an OPC stops dividing and terminally differentiates (Gao et al., 1998; Tang et al., 2000). The effect of high golli expression could be interpreted either as increased proliferation or delayed exit from the cell cycle. However, since the effect of golli on OPC proliferation disappeared after 24 h of mitogen deprivation, we interpret our results to indicate a golli-modulated enhancement of

mitosis rather than an effect on delayed exit from the cell cycle. Furthermore, our data also suggest that, after cell-cycle exit, the differentiation of both golli-KO and JOE OPCs is arrested.

The results described in the present paper indicate clearly that endogenous golli expression can modulate OPC proliferation in the presence of PDGF. No significant changes were
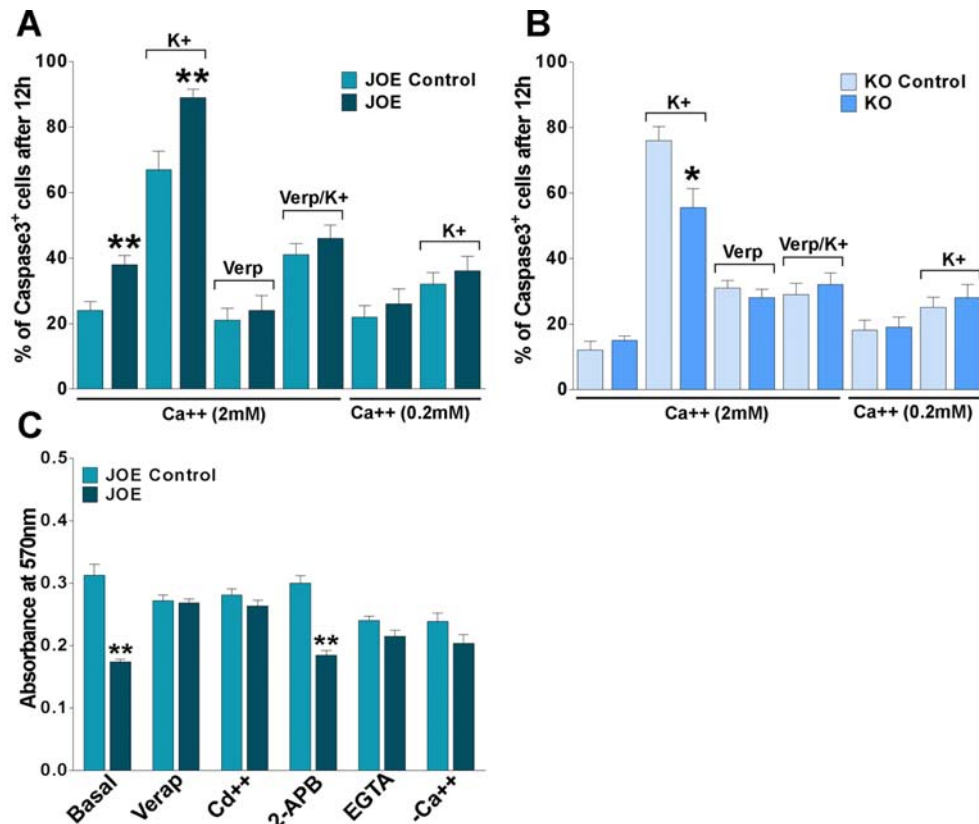
Figure 12   Ca^{2+} influx via VOCCs are involved in golli-mediated modulation of OPC death
(**A** and **B**) Real-time caspase 3 assay, using NucView^{TM} 488 caspase 3 substrate, was performed for a period of 12 h beginning at 3 DIV. Verapamil (Verp; 25 μM) and high-K^{+} (20 mM) were added to the culture medium, or extracellular Ca^{2+} concentrations were lowered from 2 mM to 0.2 mM. The effects of each treatment on golli-KO and JOE cell death were evaluated by measuring the percentage of caspase 3-positive cells as described in the Materials and methods section. Results are means ± S.E.M. for five independent experiments. *$P<0.05$, **$P<0.01$ compared with control cells. (**C**) Verapamil (Verap; 25 μM), Cd^{2+} (10 μM), 2-APB (25 μM) and EGTA (1 mM), were added to the medium in separate experiments, or extracellular Ca^{2+} concentrations were lowered from 2 to 0.2 mM (−Ca^{++}). The effects of each treatment on JOE cells viability were evaluated for a period of 12 h beginning at 3 DIV using the MTT survival assay. Results are means ± S.E.M. for two independent experiments, with duplicate cultures for each experimental condition. **$P<0.01$ compared with control cells.

found in the proliferation rate of JOE OPCs compared with genotype controls when bFGF was added separately to the culture medium. Evidence for this interpretation is shown in Figure 11(E), where, at 2 DIV, the effect of golli on OPC proliferation disappears in the absence of PDGF.

Golli mitogenic stimulation results in a biphasic Ca^{2+} response mediated by SOCCs

The BrdU-incorporation data obtained at different concentrations of PDGF (Figure 9) indicate that golli enhanced the proliferative activity of PDGF, a potent mitogen that induces OPCs to proliferate *in vitro* and can prevent premature differentiation *in vivo* (Calver et al., 1998; Noble et al., 1988; Raff et al., 1988). PDGF binds to the PDGFα receptor, which belongs to a family of tyrosine kinase receptors that has its own cytoplasmic associated tyrosine kinase activity (Taniguchi, 1995). Our findings indicate that golli produces an increase in the response of OPCs to the mitogenic stimulus

of PDGF, suggesting a novel and relevant physiological role of golli in the control of OPC proliferation and differentiation.

Few growth factors have been shown to mediate their actions via oligodendroglial Ca^{2+} signalling, with the exception of PDGF, which is a potent mitogen for OPCs and evokes oscillatory and non-oscillatory Ca^{2+} signals in cultured oligodendrocyte cell lines (Fatatis and Miller, 1997). In the present study, we have found that PDGF induced a biphasic transient increase in [Ca^{2+}]$_{int}$. The first phase occurred in the absence of external Ca^{2+}, was completely abolished by thapsigargin pre-treatment and was unaffected by verapamil, indicating that the first phase was due to Ca^{2+} release from intracellular stores. The second phase was completely abolished by the SOCC inhibitors 2-APB and MRS-1845, and was unaffected by the VOCC inhibitor verapamil, indicating that SOCCs, but not VOCCs were involved in maintaining the elevated [Ca^{2+}]$_{int}$ characteristic of this phase. The store-operated Ca^{2+} influx was higher in JOE cells, indicating that the mechanism responsible for the effect of

golli on OPC proliferation was mediated through an increase in Ca^{2+} influx through SOCCs. Recently, Cuddon et al. (2008) reported that PDGF activates store-operated Ca^{2+} entry in neuronal precursor cells, a finding that supports our data indicating that store-operated Ca^{2+} uptake is an essential component of the PDGF mitotic mechanism in OPCs.

Overstimulation of VOCCs induces apoptosis in JOE OPCs

The role of Ca^{2+} as a death trigger was discovered three decades ago in myocytes subjected to ischaemic insults (Fleckenstein et al., 1974). Since then, numerous studies have described that lethal influx of Ca^{2+} can occur in many cell types as a consequence of receptor overstimulation and exposure to cytotoxic agents (Orrenius et al., 2003). Oligodendroglial cells, like neurons, are vulnerable to Ca^{2+} overload resulting from dysregulation of ion channels or pumps, which triggers either apoptotic or necrotic cell death. Glutamate- and K^+-mediated Ca^{2+} influx has been reported to cause oligodendrocyte death and myelin disruption, and may be implicated in multiple sclerosis and ischaemia. Other studies have shown that Ca^{2+} ionophores cause retraction of membrane sheets and necrosis in cultured oligodendrocytes and induction of vesicular demyelination *in vivo* (Schlaepfer, 1977; Benjamins and Nedelkoska, 1996; Smith and Hall, 1994).

Sustained Ca^{2+} increases that overwhelm extrusion and buffering mechanisms in oligodendrocytes trigger apoptosis. Excessive Ca^{2+} uptake can activate the mitochondrial pathway of apoptosis signalling. This requires permeabilization of the outer mitochondrial membrane and the release of pro-apoptotic mitochondrial proteins such as cytochrome *c*. Ultimately, the apoptotic cascade activates proteases such as caspases and calpains, which cleave numerous substrates including key components of the Ca^{2+} signalling system such as the plasma membrane Ca^{2+}-ATPase (Paszty et al., 2002).

In the present study, we found increased apoptotic cell death in JOE cells under basal culture conditions and after high-K^+ stimulation. JOE cell death could be prevented by the VOCC inhibitor verapamil, and disappeared in the absence of Ca^{2+}, confirming that mechanisms involving Ca^{2+} influx via VOCCs are related directly to the effect of increased golli levels on OPC death. In contrast, opposite results were found in golli-KO cells; after high-K^+ treatment, golli-KO OPCs were more resistant to apoptotic death.

To date, the role of VOCCs in oligodendrocyte death remains unclear. Earlier studies in models of optic nerve or spinal cord white matter anoxia showed that VOCCs participate in CNS white matter hypoxic injury (Fern et al., 1995; Fern, 1998; Imaizumi et al., 1999). Agrawal et al. (2000) examined the role of VOCCs in mediating post-traumatic axonal conduction abnormalities in an *in vitro* model. They showed physiological and immunohistochemical evidence, suggesting that traumatic spinal cord white matter injury is associated with Ca^{2+} influx through VOCCs expressed on periaxonal astrocytes and oligodendrocytes. These studies support the notion that a sustained rise in intracellular Ca^{2+} through VOCCs is a potential trigger for OPC/oligodendrocyte cell death leading to subsequent deleterious consequences on myelination.

In conclusion, the present study has shown that golli serves as a regulating molecule that modulates oligodendrocyte proliferation and death through two different classes of Ca^{2+} channels, SOCCs and VOCCs respectively. Golli action on cell proliferation seems to be mediated by Ca^{2+} influx initiated by the growth factor PDGF. The present study has not only underscored the importance of golli on OPC differentiation, but also revealed separate Ca^{2+} signalling pathways by which OPC proliferation and death may be regulated.

REFERENCES

Agrawal SK, Nashmi R, Fehlings MG (2000) Role of L- and N-type calcium channels in the pathophysiology of traumatic spinal cord white matter injury. Neuroscience 99:179–188.

Agresti C, D'Urso D, Levi G (1996) Reversible inhibitory effects of interferon-γ and tumour necrosis factor-α on oligodendroglial lineage cell proliferation and differentiation *in vitro*. Eur J Neurosci 8:1106–1116.

Amur-Umarjee S, Phan T, Campagnoni AT (1993) Myelin basic protein mRNA translocation in oligodendrocytes is inhibited by astrocytes *in vitro*. J Neurosci Res 36:99–110.

Benjamins JA, Nedelkoska L (1996) Release of intracellular calcium stores leads to retraction of membrane sheets and cell death in mature mouse oligodendrocytes. Neurochem Res 21:471–479.

Berridge MJ (1995) Calcium signalling and cell proliferation. BioEssays 17:491–500.

Berridge MJ, Bootman MD, Lipp P (1998) Calcium: a life and death signal. Nature 395:645–648.

Calver AR, Hall AC, Yu WP, Walsh FS, Heath JK, Betsholtz C, Richardson WD (1998) Oligodendrocyte population dynamics and the role of PDGF *in vivo*. Neuron 20:869–882.

Campagnoni AT, Pribyl TM, Campagnoni CW, Kampf K, Amur-Umarjee S, Landry CF, Handley VW, Newman SL, Garbay B, Kitamura K (1993) Structure and developmental regulation of *Golli-mbp*, a 105-kilobase gene that encompasses the myelin basic protein gene and is expressed in cells in the oligodendrocyte lineage in the brain. J Biol Chem 268:4930–4938.

Casaccia-Bonnefil P, Liu A (2003) Relationship between cell cycle molecules and onset of oligodendrocyte differentiation. J Neurosci Res 72:1–11.

Colwell CS (2000) Circadian modulation of calcium levels in cells in the suprachiasmatic nucleus. Eur J Neurosci 12:571–576.

Cuddon P, Bootman MD, Richards GR, Smith AJ, Simpson PB, Roderick HL (2008) Methacholine and PDGF activate store-operated calcium entry in neuronal precursor cells via distinct calcium entry channels. Biol Res 41:183–195.

Estacion M, Mordan L (1993) Competence induction by PDGF requires sustained calcium influx by a mechanism distinct from storage-dependent calcium influx. Cell Calcium 14:439–454.

Fatatis A, Miller RJ (1997) Platelet-derived growth factor (PDGF)-induced Ca^{2+} signaling in the CG4 oligodendroglial cell line and in transformed oligodendrocytes expressing the β-PDGF receptor. J Biol Chem 272:4351–4358.

Feng JM, Hu YK, Xie LH, Colwell CS, Shao XM, Sun XP, Chen B, Tang H, Campagnoni AT (2006) Golli protein negatively regulates store depletion-induced calcium influx in T cells. Immunity 24:717–727.

Fern R, Ransom BR, Waxman SG (1995) Voltage-gated calcium channels in CNS white matter: role in anoxic injury. J Neurophysiol 74:369–377.

Fern R (1998) Intracellular calcium and cell death during ischemia in neonatal rat white matter astrocytes *in situ*. J Neurosci 18:7232–7243.

Filipovic R, Rakic S, Zecevic N (2002) Expression of Golli proteins in adult human brain and multiple sclerosis lesions. J Neuroimmunol 127:1–12.

Filipovic R, Zecevic N (2005) Lipopolysaccharide affects Golli expression and promotes proliferation of oligodendrocyte progenitors. Glia 49:457–466.

Fleckenstein A, Janke J, Doring HJ, Leder O (1974) Myocardial fiber necrosis due to intracellular Ca overload: a new principle in cardiac patho-physiology. Recent Adv Stud Cardiac Struct Metab 4:563–580.

Gao FB, Apperly J, Raff M (1998) Cell-intrinsic timers and thyroid hormone regulate the probability of cell-cycle withdrawal and differentiation of oligodendrocyte precursor cells. Dev Biol 197:54–66.

Goto H, Tomono Y, Ajiro K, Kosako H, Fujita M, Sakurai M, Okawa K, Iwamatsu A, Okigaki T, Takahashi T, Inagaki M (1999) Identification of a novel phosphorylation site on histone H3 coupled with mitotic chromosome condensation. J Biol Chem 274:25543–25549.

Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.

Hicks GL, Franklin RJM (1999) Distinctive patterns of PDGF-A, FGF-2, IGF-1 and TGF-β1 gene expression during remyelination of experimentally-induced spinal cord demyelination. Mol Cell Neurosci 14:153–168.

Imaizumi T, Kocsis JD, Waxman SG (1999) The role of voltage-gated Ca^{2+} channels in anoxic injury of spinal cord white matter. Brain Res 817:84–92.

Jacobs EC, Pribyl TM, Feng JM, Kampf K, Spreuer V, Campagnoni C, Colwell CS, Reyes SD, Martin M, Handley V, Hiltner TD, Readhead C, Jacobs RE, Messing A, Fisher RS, Campagnoni AT (2005) Region-specific myelin pathology in mice lacking the golli products of the myelin basic protein gene. J Neurosci 25:7004–7013.

Kahl CR, Means AR (2003) Regulation of cell cycle progression by calcium/calmodulin-dependent pathways. Endocr Rev 24:719–736.

Mallon BS, Shick HE, Kidd GJ and Macklin WB (2002) Proteolipid promoter activity distinguishes two populations of NG2-positive cells throughout neonatal cortical development. J Neurosci 22:876–885.

Martin M, Reyes SD, Hiltner TD, Givogri MI, Tyszka JM, Fisher R, Campagnoni AT, Fraser SE, Jacobs RE, Readhead C (2007) T_2-weighted μMRI and evoked potential of the visual system measurements during the development of hypomyelinated transgenic mice. Neurochem Res 32:159–165.

Michel S, Itri J, Colwell CS (2002) Excitatory mechanisms in the suprachiasmatic nucleus: the role of AMPA/KA glutamate receptors. J Neurophysiol 88:817–828.

Moscarello MA, Mak B, Nguyen TA, Wood DD, Mastronardi F, Ludwin SK (2002) Paclitaxel (Taxol) attenuates clinical disease in a spontaneously demyelinating transgenic mouse and induces remyelination. Mult Scler 8:130–138.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63.

Munaron L (2002) Calcium signaling and control of cell proliferation by tyrosine kinase receptors. Int J Mol Med 10:671–676.

Noble M, Murray K, Stroobant P, Waterfield MD, Riddle P (1988) Platelet-derived growth factor promotes division and motility and inhibits premature differentiation of the oligodendrocyte/type-2 astrocyte progenitor cell. Nature 333:560–562.

Oh LY, Denninger A, Colvin JS, Vyas A, Tole S, Ornitz DM, Bansal R (2003) Fibroblast growth factor receptor 3 signaling regulates the onset of oligodendrocyte terminal differentiation. J Neurosci 23:883–894.

Orrenius S, Zhivotovsky B, Nicotera P (2003) Regulation of cell death: the calcium-apoptosis link. Nat Rev Mol Cell Biol 4:552–565.

Paez PM, Spreuer V, Handley V, Feng JM, Campagnoni C, Campagnoni AT (2007) Increased expression of golli myelin basic proteins enhances calcium influx into oligodendroglial cells. J Neurosci 27:12690–12699.

Paez PM, Fulton D, Colwell CS, Campagnoni AT (2008) Voltage-operated Ca^{2+} and Na^+ channels in the oligodendrocyte linage. J Neurosci Res, doi:10.1002/jnr.21938.

Paszty K, Verma AK, Padanyi R, Filoteo AG, Penniston JT, Enyedi A (2002) Plasma membrane Ca^{2+}ATPase isoform 4b is cleaved and activated by caspase-3 during the early phase of apoptosis. J Biol Chem 277:6822–6829.

Paz Soldán MM, Warrington AE, Bieber AJ, Ciric B, Van Keulen V, Pease LR, Rodriguez M (2003) Remyelination-promoting antibodies activate distinct Ca^{2+} influx pathways in astrocytes and oligodendrocytes: relationship to the mechanism of myelin repair. Mol Cell Neurosci 22:14–24.

Pribyl TM, Campagnoni CW, Kampf K, Kashima T, Handley VW, McMahon J, Campagnoni AT (1993) The human myelin basic protein gene is included within a 179-kilobase transcription unit: expression in the immune and central nervous systems. Proc Natl Acad Sci USA 90:10695–10699.

Raff MC, Lillien LE, Richardson WD, Burne JF, Noble MD (1988) Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture. Nature 333:562–565.

Reyes SD, Campagnoni AT (2002) Two separate domains in the golli myelin basic proteins are responsible for nuclear targeting and process extension in transfected cells. J Neurosci Res 69:587–596.

Reyes SD, Givogri MI, Campagnoni C, Kampf K, Handley V, Schonmann V (2003) Overexpression of the golli J37 isoform in transgenic mice results in CNS hypomyelination. Abstr Soc Neurosci, abstract no. 141.7

Schlaepfer WW (1977) Vesicular disruption of myelin stimulated by exposure of nerve to calcium ionophore. Nature 265:734–736.

Smith KJ, Hall SM (1994) Central demyelination induced *in vivo* by the calcium ionophore ionomycin. Brain 117:1351–1356.

Sperber BR, McMorris FA (2001) Fyn tyrosine kinase regulates oligoden-droglial cell development but is not required for morphological differentiation of oligodendrocytes. J Neurosci Res 63:303–312.

Suzumura A, Bhat S, Eccleston PA, Lisak RP, Silberberg DH (1984) The isolation and long-term culture of oligodendrocytes from newborn mouse brain. Brain Res 324:379–383.

Tang DG, Tokumoto YM, Raff MC (2000) Long-term culture of purified postnatal oligodendrocyte precursor cells: evidence for an intrinsic maturation program that plays out over months. J Cell Biol 148:971–984.

Taniguchi T (1995) Cytokine signaling through nonreceptor protein tyrosine kinases. Science 268:251–255.

Wei Y, Yu L, Bowen J, Gorovsky MA, Allis CD (1999) Phosphorylation of histone H3 is required for proper chromosome condensation and segregation. Cell 97:99–109.

Yong VW, Sekiguchi S, Kim MW, Kim SU (1988) Phorbol ester enhances morphological differentiation of oligodendrocytes in culture. J Neurosci Res 19:187–194.

Yong VW, Cheung JCB, Uhm JH, Kim SU (1991) Age-dependent decrease of process formation is augmented by protein kinase C stimulation. J Neurosci Res 29:87–99.