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AXON CONTACT-DRIVEN SCHWANN CELL DEDIFFERENTIATION

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Abstract

Mature Schwann cells (SCs) retain dedifferentiation potential throughout adulthood. Still, how dedifferentiation occurs remains uncertain. Results from a variety of cell-based assays using in vitro cultured cAMP-differentiated and myelinating SCs revealed the existence of a novel dedifferentiating activity expressed on the surface of dorsal root ganglion (DRG) axons. This activity had the capacity to prevent SC differentiation and elicit dedifferentiation through direct SC-axon contact. Evidence is provided showing that a rapid loss of myelinating SC markers concomitant to proliferation occurred even in the presence of elevated cAMP, a signal that is required to drive and maintain a differentiated state. The dedifferentiating activity was a membrane-bound protein found exclusively in DRG neurons, as judged by its subcellular partitioning, sensitivity to proteolytic degradation and cell-type specificity, and remained active even after disruption of cellular organization. It differed from the membrane-anchored neuregulin-1 isoforms that are responsible for axon contact-induced SC proliferation and exerted its action independently of mitogenic signaling emanating from receptor tyrosine kinases and mitogen-activated protein kinases such as ERK and JNK. Interestingly, dedifferentiation occurred without concomitant changes in the expression of Krox-20, a transcriptional enhancer of myelination, and c-Jun, an inhibitor of myelination. In sum, our data indicated the existence of cell surface axon-derived signals that override pro-differentiating cues, drive dedifferentiation and allow SCs to proliferate in response to axonal mitogens. This axonal signal may negatively regulate myelination at the onset or reversal of the differentiated state.

Graphical Abstract

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Keywords

cAMP; differentiation; proliferation; cell-cell interactions; cell signaling; myelination; dorsal root ganglion neurons; in vitro systems

INTRODUCTION

Mature myelinating Schwann cells (SCs) are among the few known examples of somatic cells known to undergo dedifferentiation, which is a phenomenon that typically occurs after nerve injury. Dedifferentiation allows SCs to support axonal growth, repopulate regenerated axons and in turn, form new myelin to restore nerve function (Chen et al. 2007). In fact, nerve regeneration does not occur if SCs fail to lose their myelin and undergo the series of changes that allow them to become competent to self-renew (Jessen and Mirsky 2016).

From the mechanistic perspective, the prevailing notion is that dedifferentiation occurs in response to specific extracellular signals, as both the reversal of the differentiated characteristics and the re-entry into the cell cycle require transcription factors that are sensitive to intracellular signal transduction cascades of diverse nature (Jopling et al. 2011). Among these transcription activators, c-Jun/AP1, Notch and Pax-3 have been assigned a role in mediating the loss of myelin integrity that is typically associated with injury-induced SC dedifferentiation (Arthur-Farraj et al. 2012; Doddrell et al. 2012; Woodhoo et al. 2009). Yet, the nature of the extracellular signals that drive the conversion of myelinating SCs into the immature cells are still unclear.

Recent data has indicated that transcription factors that prevent SC differentiation into myelin-forming cells, typically referred to as negative regulators of myelination, share in common their ability to drive dedifferentiation by counteracting pro-myelinating signals such as cyclin adenosine monophosphate, cAMP, (Jessen and Mirsky 2008). cAMP is sufficient to potently induce myelin gene expression and allow SCs to transition into a myelinating state (Bacallao and Monje 2015). By using isolated cAMP-differentiated SCs as a simplified *in vitro* model of the myelinating SC we found that the removal of the cAMP stimulus rather than the activation of receptor tyrosine kinases (RTKs) by growth factors such as neuregulin is sufficient to revert the differentiated characteristics and confer self-renewal capability (Monje et al. 2010). In continuing our search for candidate modulators of SC differentiation using cAMP-differentiated SCs we unexpectedly came across the finding that active dedifferentiation was dependent upon the availability of factors expressed on the surface of sensory axons from the dorsal root ganglia (DRG). As such, we herein report the existence of a novel activity bound to the DRG axolemma that not only prevents the differentiation of axon-related SCs but also drives their dedifferentiation by overriding the action of cAMP and Krox-20/Egr-2, a cAMP responsive transcription factor that is considered a master regulator of myelination (Topilko et al. 1994). We arrived to the concept of axon contact-driven SC dedifferentiation after performing a series of cell-based assays designed to evaluate the effect of both live and disrupted DRG neurite preparations on the expression of markers typical of differentiated (myelinating) SCs. We found the dedifferentiating axonal component to be a membrane-bound protein specifically expressed

in DRG neurons. This component was different from the axonal growth factors responsible for axon contact-induced SC proliferation. It was active on its own, as it did not require membrane integrity or activity to drive dedifferentiation, and allowed SCs to dedifferentiate independently of RTK activation, c-Jun upregulation and mitogen-activated protein kinase (MAPK) signaling. Based on the abovementioned functional description, we reasoned that the herein described axonal dedifferentiating component encompassed the major anticipated requirements for a juxtacrine signal that negatively regulates SC differentiation and possibly myelination.

MATERIALS AND METHODS

Materials, reagents and cell lines

N62'-O-Dibutyryl adenosine-3',5'-cyclic monophosphate (db-cAMP) and 8-(4-Chlorophenylthio) adenosine-3',5'-cyclic monophosphate (CPT-cAMP) were from Biolog (Diego, CA). Recombinant heregulin- $\beta 1^{177-244}$, a soluble peptide consisting of the EGF homology domain of $\beta 1$ -heregulin (hereafter referred to as "neuregulin") was from Genentech (South San Francisco, CA). Defined fetal bovine serum (FBS) was from HyClone (Logan, UT). Forskolin, cholera toxin, anti-CNPase (2',3'-cyclic-nucleotide 3'-phosphodiesterase) and anti- β -actin were from Sigma (St. Louis, MO). S100 and glial fibrillar acidic protein (GFAP) antibodies were from DAKO (Carpinteria, CA). Myelin-associated glycoprotein (MAG) and myelin protein zero (P₀) antibodies were from Chemicon (Temecula, CA). Vimentin, N-cadherin and phosphorylated-Akt, -ERK, -ErbB3 antibodies were from Cell Signaling (Beverly, MA). ErbB3, p75^{NGFR} and c-Jun antibodies were from Santa Cruz Biotech (Santa Cruz, CA). Minichromosome maintenance protein-2 (MCM2) antibodies were from Bethyl Lab (Montgomery, TX). Anti-nestin was from EMD-Millipore (Darmstadt, Germany). Anti-peripheral myelin protein-22 (PMP22) was from AbCam (Cambridge, UK). U0126 was from Promega Corp. (WI, USA). SP600125 was from Biomol (Plymouth Meeting, PA). The tyrosine kinase inhibitors EGFR/ErbB2 (4557W), AG825 (tyrphostin), genistein and PP2 were from Calbiochem-Novabiochem Corp. (La Jolla, CA). Bromodeoxy-uridine (BrdU) antibody and DNAase were from Amersham (Piscataway, NJ). Thy-1.1 and p75^{NGFR} (clone 192) hybridoma cells were from American Type Culture Collection (ATCC, Manassas, VA). The O1 and O4 cell lines were donated by Dr. Mellita Schachner. Krox-20 and periaxin (Prx) antibodies were kindly provided by Dr. Dies Meijer and Peter Brophy, respectively.

Primary cultures of nerve-derived human Schwann cells and fibroblasts

Adult rat sciatic nerve SCs were isolated from pre-degenerated explants, purified and expanded by a modification of a previously reported method (Morrissey et al. 1991), as described recently (Bacallao and Monje 2015). SC cultures consisted of >98% SCs based on immunodetection of S100 and p75^{NGFR}. Experiments were performed using early passage SCs (2–4 rounds of expansion) plated on poly-L-lysine (PLL)-laminin coated 24-well dishes. SCs obtained by this method are competent to differentiate in response to cAMP and form myelin upon co-culture with DRG neurons (Bacallao and Monje 2015).

Adult sciatic nerve fibroblasts were established from the adherent populations that migrated out of the degenerating nerve explants. Fibroblasts were cultured on a PLL substrate and subjected to one round of expansion in DMEM-10% FBS. Cultures consisted of >90% Thy1.1 positive cells.

Cultures of purified embryonic neurons from the dorsal root ganglia and the spinal cord

Embryonic day-15 dissociated DRG neurons were established, purified and maintained as described recently (Bacallao and Monje 2015). Typically, 20,000–30,000 total cells (in 40–50 μ l of medium) from enzymatically dissociated DRG bodies were placed in the center of a laminin-coated 24-well dish in Neurobasal medium containing B27 supplement, 10 ng/ml nerve growth factor and 1 mM L-glutamine. Non-neuronal cells were removed by a 3-day treatment with 5-fluoro-2'-deoxyuridine (10 μ M). This method allows the development of a system consisting of purified neurons localizing at high density in a small area in the center of the well. The axonal outgrowth extends radially and spans throughout the available surface. Exceptions included low density cultures which used <10,000 neuronal cells/drop as initial plating density and cultures established with an eccentric location of the droplet to create axon-free areas at the edge of the culture system.

Embryonic day-15 rat spinal cord neurons were obtained and purified of contaminating cells essentially as described for DRG neurons. Purified spinal cord neurons did not contain motoneurons, as judged by p75^{NGFR} immunodetection. For cell re-plating experiments (i.e. enzymatic dissociation of the purified neuronal cultures), preparation of cell homogenates and isolation of sub-cellular fractions, neurons were plated as evenly dispersed 50 μ l droplets onto PLL-coated 10-cm dishes (Andersen et al. 2016). DRG and spinal cord neurons were used for experimentation at 15–21 days and 10–12 days after initial plating, respectively, due to the restricted survival of the spinal cord cells.

Cell lines

Stable HEK293 cells overexpressing the cDNAs of neuregulin-1 type I and III (Zhang et al. 2006) were a kind donation of Drs. Zhiyou Zhang and David Carey (Geisinger Clinic, Danville, PA). Wild type HEK293 cells (ATCC) were used as controls, as these cells do not possess neuregulin activity. HEK293 cells were cultured in PLL-coated dishes in DMEM containing 10% FBS, gentamycin and geneticin sulfate. PC12 cells (ATCC) were cultured in PLL/laminin-coated dishes in 10% FBS-supplemented RPMI-1640 medium containing gentamycin.

Preparation of cell homogenates and subcellular fractions under native conditions

Cultures were rinsed with ice-cold Hank's balanced salt solution (HBSS) without calcium or magnesium, detached by scraping and collected by low speed centrifugation prior to mechanical disruption by repeated passages through the needle of a tuberculin syringe (in ice-cold HBSS). Homogenates were cleared off debris by low speed centrifugation after inspection by phase-contrast microscopy to guarantee the absence of intact neuronal somata (or intact cells). The resultant supernatant was used as such for experimentation (referred to as 'disrupted neurons' in the text and figures) or further centrifuged (40,000 x g) to collect cytosolic (supernatant; referred to as DRG cytosol) and membrane-enriched fractions (pellet;

referred to as DRG membranes). Fractions were used immediately after collection or snap frozen in liquid nitrogen. Both the disrupted DRG neurons and the membrane preparations derived therefrom contained the axon-bound mitogenic activity. Most stimulation experiments used unfractionated, cleared DRG neuron homogenates based on the ease of preparation and empirical data showing retention of the biological activity present in neurites (Salzer et al. 1980b). The mitogenic activity of preparations from DRG neurons and cell lines was determined by means of [³H]-thymidine incorporation assays and Western blot analysis of neuregulin/ErbB activation in undifferentiated SCs. Fractions do not lose their mitogenic (or dedifferentiating) activity after one cycle of freezing-thawing.

To prepare trypsinized membranes, DRG neuron homogenates were incubated in calcium/magnesium-free phosphate buffer saline containing trypsin (30 µg/ml) for 50 min at 30°C. The trypsin activity was stopped by placing the tubes on ice and collecting the membranes by centrifugation at 40,000 x g. Control samples were processed identically with the exception of the inclusion of trypsin in the incubation buffer. All fractions used for stimulation contained equal total protein (typically 10–20 µg).

Proliferation assays

The incorporation of [³H]-thymidine or the thymidine analog BrDU into nuclear DNA was assayed as a measure of S-phase entry. Briefly, mitogen and serum-starved SCs (100,000 cells/well) were exposed to medium containing 0.25 µCi/well [³H]-thymidine (Perkin-Elmer) or BrDU (1 µM) under the conditions described in the figure legends. In some experiments, neuregulin (10 nM) was used in combination with a mitogenic dose of forskolin (2 µM) and/or FBS (10%) to achieve a maximal response in SC proliferation. The incorporated tritium (liquid scintillation counting) or BrDU (immunofluorescence microscopy) was determined 2–3 days after stimulation, as described in (Monje et al. 2010). The expression of the nuclear G1/S marker MCM2 (Kato et al. 2003) was used as alternative measure of proliferation.

SC proliferation induced by contact with live axons was determined by plating purified SCs onto cultures of purified DRG neurons (established in 24-well dishes), as SCs readily undergo mitosis upon contact with sensory neurites in the absence of added mitogenic factors. For co-culture initiation, sub-confluent cultures of mitogen and serum-starved SCs were trypsin-dissociated to obtain a single cell suspension that was immediately seeded (usually 100,000 cells) in a drop-by-drop manner onto the neuronal cells in B27-supplemented Neurobasal medium (a medium formulation non-supportive of SC proliferation). To prevent possible detrimental effects of the treatments on the physical interaction between SCs and axons, which is a crucial prefatory step for proliferation, SCs were allowed to attach to and extend processes along the axonal surface for 4 h prior to the addition of cAMP-stimulating agents and/or kinase inhibitors. [³H]-thymidine or BrDU were added at the time of stimulation and maintained throughout the time course of experimentation.

Differentiation and dedifferentiation assays

The cell permeable analog CPT-cAMP was provided at a final concentration of 250 μ M to both induce and maintain a state of differentiation. Other cAMP-stimulating agents, including forskolin (20 μ M), cholera toxin (200 ng/ml) and db-cAMP (1 mM) were used in replacement of CPT-cAMP to maintain SC differentiation (Monje et al. 2010). Cells were analyzed for the expression of phenotypic markers, or re-plated for use in experimentation, 3–4 days after treatment initiation, which is the minimum time required for SCs to achieve a differentiated phenotype expressing high levels of myelin markers (Monje et al. 2009). Differentiation assays were carried out in DMEM-1% FBS medium, which supports the differentiation but not the proliferation of isolated SCs. In some experiments, CPT-cAMP-containing medium was supplemented with 10% FBS to support the survival and differentiation of isolated SCs. Cultures maintained from the outset in the absence of CPT-cAMP served as controls for undifferentiated, immature cells.

To assess SC dedifferentiation *in vitro*, SCs were induced to differentiate as described above, and then subjected to the potentially dedifferentiating treatments in medium containing CPT-cAMP unless otherwise noted. Two independent and complementary strategies were used to assess SC dedifferentiation: (1) Plating a suspension of cAMP-differentiated SCs onto cultures of purified neurons; or alternatively, (2) Plating a suspension of purified DRG neurons (live or disrupted) onto cultures of cAMP-differentiated SCs. For the first strategy, cAMP-differentiated SCs established in 10-cm dishes ($2-3 \times 10^6$ cells/plate) were trypsin-dissociated and re-plated onto cultures of purified DRG neurons (SC-neuron cultures) or laminin-coated dishes (SC-only cultures, control). For the second strategy, purified DRG neurons were trypsin-dissociated to obtain a single cell suspension (live neurons) or mechanically disrupted to obtain a neuronal homogenate (and/or sub-cellular fractions) prior to being used to stimulate cAMP-differentiated SCs. SCs were analyzed for the expression of phenotypic markers (by immunofluorescence microscopy and Western blot), and/or S-phase entry over a time period of up to 3 days. Removal of CPT-cAMP (referred to as -cAMP in the figures) was used a known dedifferentiating stimulus (Monje et al. 2010). To simplify the interpretation of results, proliferation and differentiation assays were done in parallel cultures subjected to identical experimental conditions.

Immunofluorescence microscopy was carried out as described in (Bacallao and Monje 2015). SC-specific markers were used as follows. Krox-20, O1, MAG, P₀, CNPase, Prx, and PMP-22 were used as markers of the differentiated, myelinating SC phenotype. GFAP, c-Jun, p75^{NGFR}, N-Cadherin, nestin, and vimentin were used as markers of the immature, non-myelinating SC phenotype. S100 and O4 (sulfatide) were used as general SC-specific markers. SC-neuron cultures were routinely stained with neurofilament antibodies (neuronal/axonal marker) and DAPI (nuclear marker), which served as reference for the extension of the axonal web and the location of the cells, respectively. For cell quantification analysis, pictures from random fields were taken at low magnification and the proportion of cells that labeled positive for the indicated markers was determined in reference to the total number of cells (DAPI staining). At least 1,000 cells were analyzed in each sample. Cells were classified as positive or negative for the expression of Krox-20 (nuclear staining), O1/Prx (membrane staining), and BrDU/MCM2 (nuclear staining) in comparison to non-treated

controls and without regard to the variability of staining intensity shown by individual cells. For cell surface labeling with O1 antibodies, only those cells that exhibited homogeneous staining on the cell membrane were counted as positive. Immature, proliferative SCs were devoid of specific O1 staining.

Statistical significance was analyzed relative to each control group by a one-way analysis of variance followed by a post-hoc Tukey test using IBM SPSS statistics software. Triplicate samples from each experimental condition were analyzed. A p-value <0.05 was interpreted as statistically significant.

Isolation of myelinating SCs by magnetic-activated cell sorting

For co-culture initiation, SCs (2.5×10^6 cells) were seeded onto purified DRG neuron cultures established in PLL-coated 10-cm plates. SCs were allowed to re-populate the axonal outgrowth for 10–12 days before inducing myelination by the addition of L-ascorbic acid (50 $\mu\text{g/ml}$) and 5% FBS. For magnetic-activated cell sorting, heavily myelinated (5 week-old) SC-neuron cultures were enzymatically dissociated using 0.1 % collagenase (40 min) and 0.25 % trypsin (30 min). Cells were collected and filtered to obtain a homogeneous single cell suspension prior to incubation with Myelin Removal beads (Miltenyi Biotec, Germany) and separation through MS-columns as per the manufacturer's recommendations. The total (mixed populations), eluted (enriched in non-myelinating cells) and retained (enriched in myelinating cells) fractions were counted and plated in multi-well dishes for stimulation and analysis by immunofluorescence microscopy, as reported in (Andersen et al. 2016).

Western blots

Protein samples were prepared, subjected to electrophoresis and transferred to polyvinylidene fluoride membranes as described previously (Bacallao and Monje 2015). Gels were loaded with equal protein samples (usually 5 μg protein/lane) as determined by using the Pierce™ 660nm Protein Assay Reagent. Membranes were blocked, incubated overnight with primary antibodies (1:500–1:1,000) and for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000). Immunoreactive bands were detected by enhanced chemiluminescence (ECL) using ECL Advanced or ECL Plus (GE Healthcare) depending on signal intensity. Whenever appropriate, the expression of ErbB3 (or β -actin) is shown as a control for the equal loading of protein samples. ErbB3 is a SC-specific marker that is not expressed by DRG neurons or fibroblasts. The total levels of ERK and Akt expression served as a control for the treatments inducing ERK and Akt phosphorylation, respectively. Densitometric quantification of Western blot profiles was done using Image Studio Lite Software (version 5.2).

Detection of cell surface lipids by live cell immuno-labeling

The cell surface lipid antigens O1 and O4 were detected by live cell labeling (20 min, RT) with hybridoma culture supernatant produced *in house*. Cell labeling was followed by fixation for immunofluorescence microscopy analysis or lysis for analysis by a modified Western blot protocol (Monje et al. 2010). The O1/O4 immunoglobulins (IgM type) were detected by ECL after incubating the blocked PVDF membranes with anti-mouse-HRP.

Western blot profiles shown in the figures display the ~30 kDa molecular weight bands corresponding to the light chains of the IgMs.

RESULTS

Factors expressed by DRG neurons prevent SC differentiation in response to cAMP

It is well-established that contact with sensory axons from the DRG vigorously induce SC proliferation at least in part through the expression of neuregulin isoforms anchored to the axonal surface (Ratner et al. 1988; Wood and Bunge 1975). Hence, to investigate the effect of axonal mitogens on cAMP-induced SC differentiation, we subjected axon-related SCs (and isolated SCs for comparison) to stimulation with CPT-cAMP before determining the expression of markers of differentiation. For these experiments, SCs were plated as a single cell suspension onto cultures of dissociated DRG neurons (SC-Neuron) and laminin-coated dishes (SC-only), in the absence (control) or presence of CPT-cAMP for 3 days. Prolonged stimulation of isolated SCs with this highly permeable analog of cAMP promptly induces myelin gene expression and cell cycle exit. Laminin was used because it provides an effective substrate for SC adhesion without changing the expression of markers of differentiation or inducing proliferation (Monje et al. 2009). In addition, the medium in SC-only cultures was supplemented with combined soluble neuregulin and serum so as to mimic the strong mitogenic environment provided by the DRG axons.

As shown in Figure 1A–B, SCs plated on a bed of axons, but not on a laminin substrate, were impaired to respond to CPT-cAMP by increasing the expression of O1 (galactocerebroside), which is a reliable marker for the SC's transition into a differentiated, post-mitotic state. Axon-related SCs not only failed to express O1 in response to cAMP but also continued to proliferate upon contact with axons, as judged by the incorporation of BrDU into nuclear DNA (Figure 1A, upper panels, and C). On the contrary, isolated SCs effectively differentiated and did not proliferate despite the presence of soluble mitogens (Figure 1A, lower panels, and C). This result suggests that the DRG neurons themselves, rather than an environment supportive of proliferation, precluded cAMP-induced differentiation.

To confirm this view, we next stimulated isolated SCs with CPT-cAMP in the absence (control) or presence of a mitogenically active neuronal homogenate consisting of all subcellular fractions, including the neurites. To obtain this homogenate, purified DRG neurons were subjected to mechanical disruption under native conditions and cleared by centrifugation. The resultant preparation (referred to as disrupted neurons in the figures) was added directly to the culture medium as extracellular stimulus. Results indicated that in the presence of a neuronal homogenate and CPT-cAMP, SCs not only remained undifferentiated, as evidenced by their failure to acquire O1 expression (Figure 2A, upper panels, and B, left graph) but also proliferated vigorously, as evidenced by the high percentage of cells that incorporated BrDU (Figure 2A, lower panels, and B, right graph). Western blot analysis further confirmed that these conditions prevented O1 expression and increased the expression of the G1-S marker MCM2 while still allowing SCs to enhance the expression of Krox-20 and decrease that of c-Jun and GFAP (Figure 2C), which are two well-known markers of immature SCs.

To corroborate that the disrupted DRG neuron preparations retained the mitogenic and ErbB-inducing activities typical of live DRG neurons, we performed [³H]-thymidine incorporation assays (Figure 2E) and Western blot experiments that measured the activation of pathways downstream of the ErbB (neuregulin) receptor (Figure 2D). The disrupted neuronal preparation strongly promoted SC proliferation (Figure 2E) along with the phosphorylation of ErbB3, ERK and Akt on key activating residues in a manner similar to a bioactive 10 nM solution of a neuregulin peptide consisting of the conserved EGF-like motif of heregulin β -1 (Figure 2D). The neuronal preparation was insufficient on its own to drive the expression of Krox-20, reduce the expression of c-Jun or promote other changes associated with differentiation (Figure 2C and F). Altogether, these results suggest that DRG neurons express a component that antagonizes cAMP-induced differentiation. Strikingly, the potency of DRG neurons to inhibit SC differentiation was maintained even after disruption of cellular organization.

cAMP-differentiated SCs undergo dedifferentiation upon contact with DRG axons even in the presence of cAMP-stimulating agents

In isolated SCs, persistent cAMP elevation is required to maintain a state of differentiation because the simple removal of the cAMP stimulus allows SCs to rapidly dedifferentiate, i.e. lose the expression of myelinating SC markers such as O1, MAG and P₀, independently of the cells transitioning into the S-phase. Changes in differentiation are reversible only upon cAMP withdrawal, as soluble mitogens provided alone or in combination are not sufficient to induce dedifferentiation (Monje et al. 2010). Nevertheless, the observations presented in Figures 1 and 2 suggested the intriguing possibility that neurons/axons would exert an antagonistic effect not only on the initiation but also the maintenance of a differentiated state. To assess whether live axons would induce dedifferentiation, cAMP-differentiated SCs (referred to as dSCs in the figures) were trypsin-dissociated and seeded as a single cell suspension onto purified DRG neurons (SC-neuron) or a laminin substrate (SC-only) in the absence or presence of CPT-cAMP. Cultures were analyzed for the expression of markers specific to the myelinating SC phenotype by means of immunofluorescence microscopy (Figure 3A–B) and Western blot (Figure 3C). Sibling cultures were analyzed for S-phase entry by means of BrDU incorporation (Figure 4A), [³H]-thymidine incorporation (Figure 4C–D), quantification of S100 positive mitotic figures (Figure 4B) and Western blot analysis of MCM2 expression (Figure 3C). Undifferentiated SCs (referred to as SCs in the figures) were used as controls, as they exhibit negligible expression of myelin markers and proliferate extensively in response to axonal growth factors. These experiments were carried out over a time period of 24 to 72 hours after co-culture initiation, as specified in the figure legends.

Results indicated that cAMP-differentiated SCs plated on an axonal substrate lost the expression of O1 (Figure 3A–C), P₀ (Figure 3C) and MAG (Figure 1B, Supplementary) shortly after the onset of co-culture. Staining of SCs with the cytoplasmic marker S100 (Figure 3A, middle panels) and the membrane marker O4 (Figure 1A, Supplementary), two well-recognized SC-specific markers, revealed that axon-related cAMP-differentiated SCs rapidly (within 3 days) acquired the roughly elongated shape that is often correlated with the immature (proliferative) state. Elevation of cAMP by means of CPT-cAMP somehow

delayed the acquisition of the elongated shape in SC-neuron cultures initiated with differentiated SCs (Figure 3A, lower panels, and 1A, Supplementary). Yet, CPT-cAMP treatment failed to maintain high levels of O1 and MAG expression in those SCs that established contact with the axons (Figures 3A–B, and 1B, Supplementary).

Results were similar regardless of the type of cAMP-stimulating agent provided in the culture medium. As shown in Figure 3D–E, the use of db-cAMP (a potent cell permeable cAMP derivative), cholera toxin (an activator of the adenylyl cyclase G α stimulatory subunit) and forskolin (a direct transmembrane adenylyl cyclase activator) supported high levels of myelin gene expression in isolated but not axon-related SCs (shown only for O1 expression). Because the expression of S100 (Figure 3A) and O4 (Figure 1A, Supplementary) was maintained high in axon-related SCs, we conclude that the disappearance of myelin-related markers was likely not associated to a loss of the SC phenotype *per se*.

A striking observation was that axon-related, cAMP-differentiated SCs resumed proliferation in a manner similar to that of non-differentiated SCs (Figure 4A–C). In the absence of neurons, cAMP-differentiated SCs maintained high levels of O1 expression (Figures 3A and 4A), an enlarged phenotype, and a post-mitotic state even in the presence of soluble mitogens (Figure 4D). Images of SC-neuron cultures double-immunostained with an axonal marker (neurofilament) and a proliferation marker (BrDU) are provided to show that proliferation occurred only in axon-associated SCs regardless of their initial state of differentiation (Figure 4A). Axon-deprived SCs, i.e. cells plated in culture wells without neurons or cells residing outside of the axonal substrate, did not proliferate (Figure 4A and data not shown), as the culture medium used was non-supportive of SC proliferation. Provision of soluble mitogens to cultures of isolated SCs allowed extensive proliferation only in cultures of non-differentiated SCs (Figure 4D).

The failure of cAMP-differentiated SCs to maintain high levels of myelin gene expression upon contact with DRG axons, along with their re-entry into the cell cycle, were key indications of SC dedifferentiation. In addition to mitogenic factors, axons may provide a dedifferentiating stimulus that counterbalances the pro-differentiating action of cAMP.

An activity of DRG neurons was sufficient to induce dedifferentiation and proliferation of cAMP-differentiated SCs

We next tested whether cAMP-differentiated SCs stimulated with either intact or disrupted (homogenized) DRG neurons could maintain high levels of O1 expression and/or a cell cycle arrested state (Figure 5). As a source for live neuronal cells, we used a single cell suspension of purified DRG neurons obtained by mild trypsinization (Figure 5A–B, middle panels). DRG neuron homogenates (prepared as described in Figure 2) were used to represent disrupted neuronal cells (Figure 5A–B, right panels). Cultures were analyzed by immunofluorescence microscopy to detect the expression of O1 and the incorporation of BrDU 3 days after treatment (Figure 5A–B). The expression of MCM2 was determined by Western blot (Figure 5C) over the course of 1 to 3 days after the onset of stimulation with disrupted neurons. Sibling cultures of non-differentiated SCs were stimulated and analyzed in parallel to provide a control for the effectiveness of treatment (Figure 5B and C, panels

labeled as SCs). Results indicated that stimulation of differentiated SCs with live or disrupted neurons effectively reduced O1 expression and induced S-phase entry even in the presence of CPT-cAMP. In Figure 5, note that the neuronal bodies promptly extended a web of axons along the SC substrate and induced proliferation by contact within 72 hours in both differentiated (Figure 5A) and non-differentiated SCs (Figure 5B). Higher magnification images are provided in Figure 5A, lower panels, showing typical examples of proliferating (BrDU positive) SCs in close association with DRG neurites.

The effectiveness of the DRG homogenate preparations to induce BrDU incorporation in differentiated and non-differentiated SCs (Figure 5A–B, right panels) was confirmed by Western blot detection of MCM2 expression and ErbB activation (shown only for ErbB3 phosphorylation), (Figure 5C). Removal of CPT-cAMP from the culture medium of cAMP-differentiated SCs reduced O1 expression to an extent similar to stimulation with disrupted DRG neurons in the presence of CPT-cAMP (Figure 5D–E). As expected, removal of cAMP reduced O1 expression without a concomitant induction of BrDU incorporation (Figure 5D). Of note, DRG axons or the fractions derived there from did not mimic the action of CPT-cAMP to induce O1 expression in cultures of undifferentiated SCs (Figure 5B).

Collectively, these experiments supported the existence of an activity in DRG neurons (rather than live neurons) with the capability to induce SC dedifferentiation. This activity not only reduced the expression of myelin-associated markers but also allowed SCs to rapidly re-enter the cell cycle in the presence of pro-differentiating cues.

An activity of DRG neurons counterbalanced the action of cAMP on the expression of myelin markers rather than immature SC markers

To investigate whether factors expressed in DRG neurons affected myelin gene expression in general, we conducted additional immunofluorescence microscopy and Western blot experiments using antibodies against various markers that define the differentiated (mature, myelinating) and undifferentiated (immature) SC phenotypes. For this, cAMP-differentiated and non-differentiated (control) SCs were treated with a bioactive preparation of disrupted purified DRG neurons in the presence of BrDU, essentially as described in Figure 5, prior to fixation or preparation of Western blot samples. Stimulation of differentiated SCs was carried out in medium containing CPT-cAMP to provide a strong stimulatory signal for differentiation during the course of treatment. As in previous experiments, BrDU incorporation assays were performed together with immunodetection of SC markers to confirm the biological activity of the preparations used (Figure 6A–B). To represent myelinating SC markers, a comparative analysis of the expression of Prx, P₀, CNPase, PMP22, and O1 using the same samples was performed. These markers were chosen because there is extensive previous data showing that their expression is upregulated by treatment with cAMP. To represent immature SC markers, samples were analyzed for the expression of N-cadherin, nestin, vimentin, GFAP and p75^{NGFR}, as these markers are known to be downregulated by cAMP treatment. The unchanged expression of ErbB3 and β -actin is shown for control purposes. Results from these experiments provided evidence that treatment with disrupted neuronal preparations reduced the levels of expression of the aforementioned myelin markers, and induced proliferation, without a concomitant increase

in the expression of immature SC markers. Based on these results, we conclude that the activity expressed in DRG neurons counteracts the effect of cAMP on maintaining high levels of myelin gene expression. Nonetheless, this activity seems not to be sufficient to drive the expression of a broad panel of immature SC markers.

A DRG neuron-specific activity induced dedifferentiation of cultured myelinating SCs

We next tested the specificity of the cellular responses after changing either the source of neurons or that of differentiated SCs in our simplified dedifferentiation assays. Firstly, to replace the source of DRG neurons as a stimulatory signal for SC dedifferentiation, we used identical homogenization methods to prepare native homogenate preparations from cultures of purified spinal cord neurons, peripheral nerve fibroblasts and the SCs themselves collected from cultures of non-differentiated and cAMP-differentiated SCs. Secondly, to replace the source of differentiated SCs we used O1 positive, myelinating SCs from SC-DRG neuron cultures established in the absence of cAMP-stimulating agents. For this purpose, we developed a magnetic-activated cell sorting method to discriminate and separate O1 positive (myelinating) and O1 negative (immature) SCs from enzymatically dissociated, heavily myelinated (ascorbate-treated) SC-neuron cultures. The O1-enriched SC populations were plated in individual wells immediately after magnetic separation and stimulated for 2 days with disrupted DRG neurons in the absence or presence of CPT-cAMP prior to detection of O1 expression.

Data presented in Figure 7A–B confirmed that preparations derived from DRG neurons, as opposed to other types of cells, diminished O1 expression in cultures of cAMP-differentiated SCs. Data presented in Figure 7C–D confirmed that DRG-treated myelinating SCs reduced O1 expression in a manner similar to cAMP-differentiated SCs. We conclude that a DRG neuron-related activity diminishes myelin gene expression through a cell-type specific mechanism of action. The sensitivity of myelinating SCs to this neuronal activity further suggests that axon-to-SC communication may underlie the onset and progression of SC dedifferentiation.

Physical contact between the membrane of the SC and the membrane of the DRG axons was required for SC dedifferentiation

To begin identifying the relevant variables that affected the loss of myelin gene expression in axon-related, cAMP-differentiated SCs, we performed additional experiments that consisted of: (1) Plating a fixed number of differentiated SCs onto cultures consisting of increasing numbers of DRG neurons (Figure 8A–B); and (2) Plating increasing numbers of differentiated SCs onto cultures containing a fixed number of DRG neurons (Figure 8E–F). We also used conditioned medium obtained from the purified DRG neurons to test whether soluble factors released by the neurons were responsible for the induction of SC dedifferentiation (Figure 8C). Results revealed that the density of the DRG neurons rather than the density of the differentiated SCs used to initiate the co-culture was the most important factor affecting the loss of a differentiated phenotype. Co-cultures established using increasing numbers of neurons indicated that the percentage of cells that maintained O1 expression inversely correlated with number of neurons and the density of axonal substrate (Figure 8A–B). Yet, in the absence of exogenously added CPT-cAMP, SCs lost the

expression of O1 regardless of the density of the neurons (Figure 8B) or the SCs used (Figure 8E–F). Two key observations supported the idea that direct physical contact of the SCs with the DRG neurites was required. Firstly, the conditioned medium of high density neuronal cultures failed to mimic the effect of live or disrupted neurons on reducing O1 expression (Figure 8C). Secondly, those differentiated SCs that did not or only poorly engaged into physical contact with the axons were able to preserve their characteristics of differentiated cells in cAMP-supplemented medium (Figure 8A and D). This was clearly illustrated by the differential levels of O1 expression in SCs located in central *versus* peripheral areas of the culture well deprived of an axonal outgrowth (Figure 8D). These results are consistent with previous transmission electron microscopy studies showing that cAMP-differentiated SCs tightly appose their plasma membranes to the DRG axolemma in a conformation that is optimal for neuron-to-SC signaling leading to mitotic cell division (Monje et al. 2009). It thus plausible that similar to proliferation, SC dedifferentiation requires direct and extensive SC-axon contact.

A trypsin-sensitive component from the DRG axons was responsible for dedifferentiation

Our results indicated that disrupted neurons were nearly as effective as the live ones to promote SC dedifferentiation and proliferation. Therefore, to dissect which sub-cellular component of the DRG neurons contained a dedifferentiating activity, we subjected the preparations of disrupted DRG neurons to a simple protocol of sub-cellular fractionation under native conditions so as to obtain membrane-enriched (particulate) and cytosolic (soluble) fractions from the neuronal cells. In turn, these fractions were used to stimulate cAMP-differentiated SCs basically as described in previous figures. An immunofluorescence microscopy analysis of O1 expression and BrDU incorporation provided evidence for a selective partitioning of the dedifferentiating activity into the membrane fraction (Figure 9A–B). The bioactivity of all fractions was further confirmed by means of [³H]-thymidine incorporation assays in non-differentiated SCs (Figure 9C). As expected based on previous findings (Salzer et al. 1980b), the resultant DRG membrane preparations exhibited the stronger mitogenic activity (Figure 9C).

We next sought out to investigate whether the dedifferentiating component of DRG axons was sensitive to proteolytic degradation, as research has shown that the membrane-bound mitogenic activity of DRG axons can easily be removed by trypsinization (Salzer et al. 1980a). Experimental data provided in Figure 9D–E revealed that treatment of cAMP-differentiated SCs with mildly trypsinized, non-mitogenic DRG membranes continued to express high levels of O1. The lack of BrDU incorporation by non-differentiated (Figure 9D, upper panels, and F) and differentiated SCs (Figure 9D–E) confirmed the loss of mitogenic activity as a consequence of trypsinization. Altogether, data suggests that the dedifferentiating effect is provided by a non-diffusible membrane protein or protein complex specifically expressed on the surface of DRG neurites.

The dedifferentiating component of DRG axons was different from the mitogenic component

The data presented above suggested that SCs exposed to an axonal environment receive at least two different types of signals through their established contact with sensory axons.

These include a set of signals that induce mitogenesis and another set of signals that prevent their differentiation and/or induce their dedifferentiation. To the best of our knowledge, an axon-derived signal exhibiting the latter properties has not yet been described. Thus, to begin addressing the nature of this novel axonal component, we designed various experiments to examine whether the dedifferentiating and mitogenic components of DRG axons would share a molecular identity (Figure 10A–D) and/or a mechanism of action in dedifferentiating SCs (Figure 10E–G).

It has been clear since the 1970s that SC proliferation requires SC-axon contact (Salzer et al. 1980a; Wood and Bunge 1975). It is also well-established that the main component that accounts for the axonal mitogenic activity is a membrane-anchored form of neuregulin. Additionally, it has been reported that cultured DRG neurons exhibit constitutive expression of several members of the neuregulin-1 family, the most prominent of which are neuregulin-1 type I and neuregulin-1 type III (Zhang et al. 2006). Because soluble neuregulin does not antagonize the effect of cAMP on inducing or maintaining a differentiated state (Monje et al. 2009; Monje et al. 2010), we tested whether the full-length neuronal isoforms of neuregulin-1 would possess a dedifferentiating activity, as the biological activity of soluble and membrane-bound neuregulin might differ considerably. Therefore, we isolated membrane fractions from two different stable HEK293T cell lines that over-express the neuronal transcripts of neuregulin-1 type I or type III. The activity of these membrane preparations was compared to that of the native DRG membranes by means of dedifferentiation assays using cAMP-differentiated SCs (Figure 10A–B). [³H]-thymidine incorporation assays (Figure 10C) and Western blot analysis of ErbB-activated signaling (Figure 10D) were used to evaluate their bioactivity in cultures of non-differentiated SCs. Even though neuregulin-enriched membranes from HEK293 cells increased SC proliferation (Figure 10C) and the activation (phosphorylation) of ErbB3, ERK and Akt to levels comparable to those elicited by DRG membranes (Figure 10D), they were apparently devoid of a dedifferentiating activity. This was judged by their inability to significantly reduce O1 expression and induce the incorporation of BrDU in cultures of differentiated SCs (Figure 10A–B). Similar results were obtained when membranes from the neural crest-derived cell line PC12, which are enriched in cell surface mitogens for SCs (Ratner et al. 1984), were used as stimuli (not shown).

Compelling data has indicated that the activation of RTKs from the ErbB family, more specifically the activation of the tyrosine kinase activity of heterodimers composed of ErbB2 and ErbB3, mediate the mitogenic response of SCs to soluble neuregulin and axon contact stimulation (Morrissey et al. 1995). The activation of ErbB2 was also shown to mediate injury-induced c-Jun up-regulation, Krox-20 down-regulation and consequent myelin loss in models of Wallerian degeneration carried out *in vitro* (Guertin et al. 2005) and *in vivo* (Shin et al. 2013). Therefore, to examine whether ErbB2 activity was required for axon-induced SC dedifferentiation, we evaluated whether inhibition of ErbB tyrosine kinase activity would antagonize DRG axon-induced O1 loss (Figure 10E–F). For this, we used a battery of pharmacological inhibitors with demonstrated activity to prevent neuregulin-induced SC proliferation without interfering with SC survival. Among these inhibitors, we included a specific inhibitor of the tyrosine kinase activity of EGFR/ErbB2 (referred to as ErbB2 inhibitor in the figure), an inhibitor of the non-receptor tyrosine kinase Src (PP2) and a

broad-spectrum tyrosine kinase inhibitor (genistein). Whereas all of these kinase antagonists effectively blocked DRG membrane-stimulated proliferation (Figure 10E–F, BrDU incorporation), none of these inhibitors prevented O1 loss (Figure 10E–F, O1 expression). The dose-dependent anti-mitogenic action of the abovementioned inhibitors was demonstrated by the reduction of neuregulin-induced [³H]-thymidine incorporation in cultures of non-differentiated SCs (Figure 10G). Other cell permeable inhibitors known to interfere with neuregulin-induced ErbB2 autophosphorylation, including AG825, effectively prevented neuregulin-dependent SC proliferation but did not antagonize axon contact-dependent O1 loss (not shown).

Cumulatively, these results indicate that a protein constituent of DRG axons, which seems to differ from neuregulin, accounts for the effect of DRG axons on SC dedifferentiation. Thus far, our data shows that axon-induced SC proliferation rather than dedifferentiation relied on ErbB activation and its downstream tyrosine kinase signaling.

Axon-induced SC dedifferentiation was independent of ERK and JNK activity, Krox-20 downregulation and c-Jun expression

It has been argued that the Krox-20/c-Jun ratio is reverted as SCs lose their myelin and dedifferentiate after injury at least in part via activation of MAPK family members. These MAPKs include the c-Jun N-terminal kinase (JNK), which is the upstream activating kinase for c-Jun, the extracellular signal-regulated kinase (ERK) and the stress-activated p38-MAPK [reviewed in (Lee et al. 2014)]. In cAMP-differentiated SCs, the Krox-20-to-c-Jun reversion occurs upon withdrawal of the cAMP stimulus (Monje et al. 2010). As shown in Figure 11A, middle lanes, and B, cAMP removal from the culture medium (referred to as – cAMP in the figure) reduced Krox-20 and restored high levels of c-Jun expression within 2 days. However, differentiated SCs that were stimulated with a freshly isolated homogenate from disrupted DRG neurons in the presence of CPT-cAMP were able to increase MCM2 expression (indicative of cell cycle re-entry) without reducing the expression of Krox-20 (Figure 11A, right lanes and B) or increasing the expression of c-Jun, GFAP (Figure 11A, right lanes) or p75^{NGFR} (Figure 11B). Similar results were obtained when DRG membranes (Figure 11C–E) or a substrate of live DRG axons (Figure 2, Supplementary) were used as stimuli. Results from immunofluorescence microscopy and Western blot experiments revealed that SCs reduced O1 expression (Figure 11C–D), increased MCM2 (Figure 11C and E) and incorporated BrDU (Figure 11E) while maintaining high levels of Krox-20 expression. Co-immunolocalization of Krox-20 along with O1 (Figure 11D), BrDU and MCM2 (Figure 11E), respectively, allowed to confirm the expression of high levels of nuclear Krox-20 in proliferating (BrDU/MCM2 positive) cells under conditions that allow a dramatic reduction of O1 expression. These results are somehow unexpected based on published data but are consistent with the observation that SCs (undifferentiated) treated from the outset with disrupted DRG neurons and CPT-cAMP express high levels of Krox-20 in the absence of O1 (Figure 2A–C).

By using pathway selective JNK (SP600125) and MEK-ERK (U0126) inhibitors, we previously showed that cAMP removal promoted c-Jun re-expression and dedifferentiation through a JNK-dependent, ERK-independent mechanism of action (Monje et al. 2010).

Therefore, we herein used SP600125 and U0126 to test whether they could prevent the loss of O1 that results from exposure to the axonal stimulus provided by disrupted (Figure 12A, upper panels, and B) or live (Figure 12A, lower panels) DRG neurons. SP600125 inhibited O1 loss and c-Jun re-appearance after cAMP removal (Figure 12A, middle panels, and B) but not in response to neurite stimulation (Figure 12A–B). In cultures of non-differentiated SCs, both SP600125 and U0126 reduced neuregulin-induced SC proliferation (Figure 12C, SC-only). Nevertheless, we found no evidence indicating that these inhibitors could prevent the disappearance of O1 or any other feature associated with dedifferentiation under our experimental conditions (Figure 12A–B). Likewise, the p38-MAPK inhibitor SB203580 supported a blockage of SC proliferation but not dedifferentiation (not shown).

To conclude, experiments using MAPK inhibitors confirmed that dedifferentiation proceeds as normal under conditions that effectively prevent S-phase progression. It was noteworthy to find that axon-driven SC dedifferentiation did not require neuregulin-initiated ErbB signaling, JNK/c-Jun activity or downregulation of Krox-20. In fact, evidence supports the concept that Krox-20 and c-Jun are sensitive to the presence/absence of cAMP rather than the axonal dedifferentiating component herein described.

DISCUSSION

Identification of a novel axonal dedifferentiating activity for SCs

Our studies uncovered the existence of a membrane-bound axonal activity with the dual capacity to prevent differentiation and induce active dedifferentiation of axon-contacting SCs. We arrived to this conclusion on the basis of experiments which used cultured differentiated SCs to assess the disappearance of myelin gene expression and subsequent S-phase entry as a function of axon contact. The first indication of the existence of an axonal dedifferentiating activity (herein referred to as ADDA) was revealed by the responses of cAMP-differentiated SCs to contact with the neurite outgrowth from DRG neurons. Somehow unexpectedly, these experiments showed that not only did axons fail to maintain the state of differentiation of axon-related SCs but also promoted a rapid reversal of the differentiated characteristics along with proliferation. This simple observation suggested that DRG neurons expressed an ADDA that complemented the activity of axonal growth factors. Results from additional experiments indicated that ADDA had the following basic properties: (1) It prevented differentiation and induced dedifferentiation by counteracting the action of cAMP and Krox-20; (2) It was a cell surface protein (or protein complex) specific to DRG neurons that exerted its action in a juxtacrine fashion; (3) It differed from membrane-bound neuregulin and induced dedifferentiation through a mechanism that was independent of the one that controlled proliferation; (4) It induced dedifferentiation without requiring ErbB/RTK or MAPK signaling; (5) It retained its bioactivity even after disruption of cellular organization; and (6) it reduced myelin gene expression in isolated myelinating cells.

Relationship between dedifferentiation and proliferation in axon-stimulated SCs

The existence of ADDA suggests that SCs are sensitive to at least two independent axon-derived activities, one activity that maintains SCs in an undifferentiated state (namely

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ADDA) and another one that drives their proliferation (namely neuregulin). Given that DRG axons express membrane-bound neuregulin and neuregulin induces demyelination *in vitro*, multiple experiments were done to compare the effect of axonal factors on SC dedifferentiation and mitogenesis, respectively. These experiments indicated that ADDA was a molecular entity different from neuregulin and that axon contact-induced SC dedifferentiation was independent of, and mechanistically uncoupled from, axon contact-induced proliferation. On one hand, ErbB2/ErbB3 activation by soluble or membrane-bound neuregulin promoted mitogenesis rather than dedifferentiation. On the other hand, ErbB and MAPK inhibition interfered with axon contact-induced SC proliferation rather than ADDA-induced dedifferentiation. The latter finding is consistent with the idea that proliferation is a secondary event with respect to dedifferentiation. Only those SCs that have lost their differentiated characteristics could progress into the S-phase.

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Given that neuregulin can have a positive or a negative outcome on myelination depending on the neuregulin isoform involved (Syed et al. 2010), we cannot rule out a role of neuregulin on dedifferentiation simply on the basis of these experiments. The effect of ADDA on SC dedifferentiation seems to be partial. This is evidenced by ADDA's ability to counteract the effect of cAMP on inducing and maintaining high levels of O1, P₀ and other myelin markers while failing on its own to decrease the expression of Krox-20, and restore that of immature SC markers in the presence of cAMP. Intriguingly, our experimental conditions allow SCs to dedifferentiate and proliferate without increasing c-Jun expression through activated JNK. c-Jun is required for SCs to adopt the molecular phenotype of the immature state both *in vitro* (Parkinson et al. 2008) and *in vivo* (Arthur-Farraj et al. 2012). Though JNK inhibition was sufficient to prevent c-Jun expression and dedifferentiation upon cAMP withdrawal, it was insufficient to prevent the loss of myelin markers in ADDA-stimulated SCs. It is currently unclear whether the maintenance of high Krox-20 levels results from impairment in c-Jun reactivation (Parkinson et al. 2004) or some other mechanism of control.

ADDA is a candidate negative regulator of SC differentiation and possibly myelination

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To the best of our knowledge, our experiments provide the first functional and biochemical evidence on the existence of a candidate negative regulator of differentiation that resides in the neurons themselves and exerts its action on SCs via cell-to-cell contact. The molecular identity of ADDA is unknown and we can only speculate its physiological role during nerve development and/or repair. It is possible that ADDA's action on halting differentiation serves to increase the SC's sensitivity to axonal growth factors as a stimulus for cell division, migration and survival during the early stages of nerve development. By interfering with myelin gene expression and/or regulating proliferation, ADDA could indirectly control the onset and duration of the myelination phase. The potential role of ADDA as a lineage specification signal for differentiation into a non-myelinating SC phenotype can be inferred by its ability to prevent myelin gene expression without changing immature SC genes. A role in nerve repair is plausible though it is particularly intriguing that ADDA initiates dedifferentiation in the context of intact axons.

The fact that reconstituted systems of SCs and DRG neurons can support effective myelination of axons and myelin breakdown followed by mitosis (after axotomy) in the absence of other types of cells (Fernandez-Valle et al. 1995) suggests that cues responsible for differentiation and dedifferentiation reside within the SC-neuron unit. It is possible that axonal signals such as ADDA contribute to the reversible transitions that SCs experience as they move into and out of a myelinating state. Whether ADDA is differentially expressed in axons during nerve development or after injury, or changes occur as axons are myelinated by SCs, is not known. We have found that myelinating SCs are sensitive to ADDA and that ADDA can maintain its activity after disruption of axonal integrity. If ADDA continues to be expressed in adult axons, a simple mechanism of injury-induced ADDA activation/release during Wallerian degeneration could contribute to injury-induced SC reprogramming. Addressing these and other questions on the physiological role of ADDA and its expression in different types of neurons is contingent upon its molecular identification and/or effective solubilization of its active component from the membrane environment.

Advantages and limitations of our simplified in vitro assays

Here, we have exploited the use of O1 positive (differentiated, post-mitotic) cells to study the mechanism of recovery of an O1 negative (immature, proliferative) state. This system recapitulates at least two critical events during SC dedifferentiation: (1) the disappearance of certain myelin-specific proteins and lipids; and (2) the re-entry into the cell cycle. Two sources of O1 positive SCs were used: (1) SCs induced to differentiate in response to cAMP in the absence of neurons; and (2) SCs induced to myelinate DRG axons in response to ascorbate treatment. The use of a single cell type (SCs) and a defined differentiating factor (cAMP) allows for a highly controlled experimental setting, thereby providing an advantage for the interpretation of mechanistic data. Provision of exogenous cAMP-stimulating agents is needed to induce and maintain high levels of myelin gene expression, including a high Krox-20/c-Jun ratio, as SCs spontaneously and autonomously dedifferentiate if cAMP is withdrawn.

These assays provide a unique tool to interrogate SCs for their requirement of extracellular dedifferentiating signals. Nevertheless, caution should be taken when extrapolating results to a physiologically-relevant model where SCs dedifferentiate in the context of degenerating axons. On one hand, we acknowledge that the state of differentiation induced by cAMP does not fully recapitulate the myelinating SC phenotype. In the absence of neurons, cAMP-differentiated SCs exhibit molecular resemblance to the pre-myelinating (O1 positive, MBP negative) phenotype. On the other hand, we acknowledge that while we have used the term dedifferentiation throughout, the observed phenotypic transitions may only recapitulate a transient phase during dedifferentiation as it happens *in vivo*. It is possible that multiple steps of mechanistically uncoupled regulatory events and context-specific signals are required as SCs progress from a myelinating to an immature (denervated) stage (Heinen et al. 2013).

Conclusions and perspectives

The unique regenerative capacity of the PNS is at least in part due to the SC's ability to dedifferentiate. Adult peripheral nerves are devoid of SC precursors. Therefore,

dedifferentiation of pre-existing mature SCs provides an effective means to generate cells with capacity to sustain nerve growth. Dedifferentiation also makes SCs vulnerable to lose their myelin upon diverse genetic and environmental insults, thus providing a basis for a variety of peripheral nerve diseases, including leprosy (Tapinos et al. 2006) and cancer (Parrinello and Lloyd 2009). Because SCs are among the few known examples of adult cells with dedifferentiation capability, they offer an invaluable opportunity to investigate basic molecular mechanisms of plasticity and cellular reprogramming in somatic cells in general. Negative regulators of differentiation represent attractive molecular targets for use in regenerative medicine as well as the development of novel therapies for peripheral neuropathies (Heinen et al. 2013). Thus, the chemical identification of ADDA and its molecular mechanism of action will open doors into investigating how negative regulators of myelination control SC differentiation during nerve development, regeneration and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Andersen ND, Srinivas S, Pinero G, Monje PV. A rapid and versatile method for the isolation, purification and cryogenic storage of Schwann cells from adult rodent nerves. *Sci Rep*. 2016; 6:31781. [PubMed: 27549422]
- Arthur-Farraj PJ, Latouche M, Wilton DK, Quintes S, Chabrol E, Banerjee A, Woodhoo A, Jenkins B, Rahman M, Turmaine M, et al. c-Jun reprograms Schwann cells of injured nerves to generate a repair cell essential for regeneration. *Neuron*. 2012; 75:633–47. [PubMed: 22920255]
- Bacallao K, Monje PV. Requirement of cAMP Signaling for Schwann Cell Differentiation Restricts the Onset of Myelination. *PLoS One*. 2015; 10:e0116948. [PubMed: 25705874]
- Chen ZL, Yu WM, Strickland S. Peripheral regeneration. *Annu Rev Neurosci*. 2007; 30:209–33. [PubMed: 17341159]
- Doddrell RD, Dun XP, Moate RM, Jessen KR, Mirsky R, Parkinson DB. Regulation of Schwann cell differentiation and proliferation by the Pax-3 transcription factor. *Glia*. 2012; 60:1269–78. [PubMed: 22532290]
- Fernandez-Valle C, Bunge RP, Bunge MB. Schwann cells degrade myelin and proliferate in the absence of macrophages: evidence from in vitro studies of Wallerian degeneration. *J Neurocytol*. 1995; 24:667–79. [PubMed: 7500122]
- Guertin AD, Zhang DP, Mak KS, Alberta JA, Kim HA. Microanatomy of axon/glial signaling during Wallerian degeneration. *J Neurosci*. 2005; 25:3478–87. [PubMed: 15800203]
- Heinen A, Lehmann HC, Kury P. Negative regulators of schwann cell differentiation-novel targets for peripheral nerve therapies? *J Clin Immunol*. 2013; 33(Suppl 1):S18–26. [PubMed: 22956147]
- Jessen KR, Mirsky R. Negative regulation of myelination: relevance for development, injury, and demyelinating disease. *Glia*. 2008; 56:1552–65. [PubMed: 18803323]
- Jessen KR, Mirsky R. The repair Schwann cell and its function in regenerating nerves. *J Physiol*. 2016; 594:3521–31. [PubMed: 26864683]

- Jopling C, Boue S, Izpisua Belmonte JC. Dedifferentiation, transdifferentiation and reprogramming: three routes to regeneration. *Nat Rev Mol Cell Biol.* 2011; 12:79–89. [PubMed: 21252997]
- Kato H, Miyazaki T, Fukai Y, Nakajima M, Sohda M, Takita J, Masuda N, Fukuchi M, Manda R, Ojima H, et al. A new proliferation marker, minichromosome maintenance protein 2, is associated with tumor aggressiveness in esophageal squamous cell carcinoma. *J Surg Oncol.* 2003; 84:24–30. [PubMed: 12949987]
- Lee HJ, Shin YK, Park HT. Mitogen Activated Protein Kinase Family Proteins and c-jun Signaling in Injury-induced Schwann Cell Plasticity. *Exp Neurobiol.* 2014; 23:130–7. [PubMed: 24963277]
- Monje PV, Rendon S, Athauda G, Bates M, Wood PM, Bunge MB. Non-antagonistic relationship between mitogenic factors and cAMP in adult Schwann cell re-differentiation. *Glia.* 2009; 57:947–61. [PubMed: 19053056]
- Monje PV, Soto J, Bacallao K, Wood PM. Schwann cell dedifferentiation is independent of mitogenic signaling and uncoupled to proliferation: role of cAMP and JNK in the maintenance of the differentiated state. *J Biol Chem.* 2010; 285:31024–36. [PubMed: 20634285]
- Morrissey TK, Kleitman N, Bunge RP. Isolation and functional characterization of Schwann cells derived from adult peripheral nerve. *J Neurosci.* 1991; 11:2433–42. [PubMed: 1869923]
- Morrissey TK, Levi AD, Nuijens A, Sliwkowski MX, Bunge RP. Axon-induced mitogenesis of human Schwann cells involves heregulin and p185erbB2. *Proc Natl Acad Sci U S A.* 1995; 92:1431–5. [PubMed: 7877996]
- Parkinson DB, Bhaskaran A, Arthur-Farraj P, Noon LA, Woodhoo A, Lloyd AC, Feltri ML, Wrabetz L, Behrens A, Mirsky R, et al. c-Jun is a negative regulator of myelination. *J Cell Biol.* 2008; 181:625–37. [PubMed: 18490512]
- Parkinson DB, Bhaskaran A, Droggiti A, Dickinson S, D'Antonio M, Mirsky R, Jessen KR. Krox-20 inhibits Jun-NH2-terminal kinase/c-Jun to control Schwann cell proliferation and death. *J Cell Biol.* 2004; 164:385–94. [PubMed: 14757751]
- Parrinello S, Lloyd AC. Neurofibroma development in NF1--insights into tumour initiation. *Trends Cell Biol.* 2009; 19:395–403. [PubMed: 19615906]
- Ratner N, Glaser L, Bunge RP. PC12 cells as a source of neurite-derived cell surface mitogen, which stimulates Schwann cell division. *J Cell Biol.* 1984; 98:1150–5. [PubMed: 6321518]
- Ratner N, Hong DM, Lieberman MA, Bunge RP, Glaser L. The neuronal cell-surface molecule mitogenic for Schwann cells is a heparin-binding protein. *Proc Natl Acad Sci U S A.* 1988; 85:6992–6. [PubMed: 3413130]
- Salzer JL, Bunge RP, Glaser L. Studies of Schwann cell proliferation. III. Evidence for the surface localization of the neurite mitogen. *J Cell Biol.* 1980a; 84:767–78. [PubMed: 6153659]
- Salzer JL, Williams AK, Glaser L, Bunge RP. Studies of Schwann cell proliferation. II. Characterization of the stimulation and specificity of the response to a neurite membrane fraction. *J Cell Biol.* 1980b; 84:753–66. [PubMed: 7358797]
- Shin YK, Jang SY, Park JY, Park SY, Lee HJ, Suh DJ, Park HT. The Neuregulin-Rac-MKK7 pathway regulates antagonistic c-jun/Krox20 expression in Schwann cell dedifferentiation. *Glia.* 2013; 61:892–904. [PubMed: 23505039]
- Syed N, Reddy K, Yang DP, Taveggia C, Salzer JL, Maurel P, Kim HA. Soluble neuregulin-1 has bifunctional, concentration-dependent effects on Schwann cell myelination. *J Neurosci.* 2010; 30:6122–31. [PubMed: 20427670]
- Tapinos N, Ohnishi M, Rambukkana A. ErbB2 receptor tyrosine kinase signaling mediates early demyelination induced by leprosy bacilli. *Nat Med.* 2006; 12:961–6. [PubMed: 16892039]
- Topilko P, Schneider-Maunoury S, Levi G, Baron-Van Evercooren A, Chennoufi AB, Seitanidou T, Babinet C, Charnay P. Krox-20 controls myelination in the peripheral nervous system. *Nature.* 1994; 371:796–9. [PubMed: 7935840]
- Wood PM, Bunge RP. Evidence that sensory axons are mitogenic for Schwann cells. *Nature.* 1975; 256:662–4. [PubMed: 1171378]
- Woodhoo A, Alonso MB, Droggiti A, Turmaine M, D'Antonio M, Parkinson DB, Wilton DK, Al-Shawi R, Simons P, Shen J, et al. Notch controls embryonic Schwann cell differentiation, postnatal myelination and adult plasticity. *Nat Neurosci.* 2009; 12:839–47. [PubMed: 19525946]

Zhang Z, Prentiss L, Heitzman D, Stahl RC, DiPino F Jr, Carey DJ. Neuregulin isoforms in dorsal root ganglion neurons: effects of the cytoplasmic domain on localization and membrane shedding of Nrg-1 type I. *J Neurosci Res.* 2006; 84:1–12. [PubMed: 16615045]

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Main Points

- A membrane-bound activity specifically expressed in sensory axons (ADDA) prevents Schwann cell differentiation and elicits dedifferentiation by overriding pro-myelinating signals such as cAMP and Krox-20.
- ADDA-induced dedifferentiation occurs independently of mitogenic signals, ErbB and cJun/JNK activity.

FIGURE 1.

Differentiation failure of axon-related SCs. SCs (undifferentiated) were plated onto DRG axons (SC-Neuron) or laminin-coated dishes (SC-only) and allowed to proliferate for 3 days prior to stimulation with CPT-cAMP (250 μ M) in the presence of BrdU labeling reagent. The medium of SC-only cultures was additionally supplemented with neuregulin (10 nM) and 10% FBS (referred to as 'mitogens') to mimic pro-mitogenic cues present in DRG neurons. Three days after CPT-cAMP administration, the cultures were evaluated for the following markers: (1) O1 (green) to reveal the state of SC differentiation; (2) BrdU (red, insets) to reveal the location of proliferating SCs; and (3) neurofilament (NF, red, upper panels) to reveal the axonal substrate. Nuclei were revealed with DAPI in this and all subsequent images (blue). The arrowheads indicate selected BrdU positive axon-related SCs. A quantification of O1 positive (differentiated) and BrdU positive (proliferating) SCs

based on image data is shown in panels B and C, respectively. In this and all subsequent figures, scale bars, 50 μ M; *, $p < 0.05$.

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FIGURE 2.

DRG neurons express a component that prevents cAMP-induced SC differentiation. **A–C.** *Blockage of SC differentiation by treatment with a preparation of disrupted DRG neurons.* Isolated SCs were left untreated (control) or treated with CPT-cAMP in the absence or presence of a mitogenically active DRG neuron homogenate (indicated as ‘disrupted DRG neurons’ in this and subsequent figures). Cells were analyzed for O1 expression (green) and BrdU incorporation (red) 3 days after treatment initiation. Representative images and quantification of results are provided in A and B, respectively. The lower panels in A are a close up view of the sections denoted by the white frames (upper panels). In C, cultures subjected to identical experimental conditions were pre-labelled with O1 antibodies prior to Western blot analysis of the indicated markers of differentiation (Krox-20, c-Jun, O1 and GFAP) and proliferation (MCM2). **D–F.** *Mitogenic and differentiating activity of the disrupted DRG neurons.* SCs (undifferentiated) were left untreated (Control, C) or treated for the indicated time points with disrupted DRG neurons, soluble neuregulin (10 nM, positive control for proliferation) or 250 μ M CPT-cAMP (positive control for SC differentiation), as indicated. The phosphorylation of ErbB3 (Tyr-1289), Akt (Thr-473), and ERK1/2 (Tyr-204) (D) are shown along with the incorporation of [3 H]-thymidine (E) to confirm the mitogenic activity of the disrupted neurons. The expression Krox-20 and c-Jun (Western blots) is shown to confirm the differentiating activity of CPT-cAMP (F). Disrupted DRG neurons promoted ErbB activation (D and F) and proliferation (E) without changing the basal levels of Krox-20 and c-Jun (C, right lanes, and F).

FIGURE 3.

Contact with live DRG axons drives SC dedifferentiation by counteracting the effect of cAMP. Undifferentiated SCs (SCs) or SCs induced to differentiate with CPT-cAMP (dSCs) were trypsin-dissociated and re-plated as a single cell suspension onto cultures of DRG neurons (SC-neuron) or laminin-coated dishes (SC-only). Experiments were carried out in the absence or presence of CPT-cAMP (A–B) or other cAMP-stimulating agents (D–E) and analyzed by immunofluorescence microscopy (A–B and D–E) and Western blot (C) using the indicated antibodies. In A–B, cultures were analyzed 40 h after co-culture initiation to reveal early changes in cell morphology and differentiation. In D–E, cultures were analyzed 72 h post-plating. Note that differentiated SCs lost the expression of O1 and re-acquired an elongated shape only when plated on DRG neurons. A quantification of O1 positive cells (B and E) is provided. Arrowheads in A (middle panel) point out to a representative S100 positive mitotic figure, indicative of cell division. Note that reduced levels of O1 and P₀ (along with increased levels of MCM2) occur without concurrent changes in O4 and ErbB3 in differentiated SCs co-cultured with DRG neurons (C, right lanes). O4 was highly

expressed in cAMP-differentiated SCs, as expected based on its well-known sensitivity to cAMP stimulation.

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FIGURE 4.

cAMP-differentiated SCs undergo proliferation upon contact with live DRG axons. Single cell suspensions of undifferentiated (SCs) and cAMP-differentiated cells (dSCs) were plated and stimulated as described in Figure 3 (legend). Proliferation was determined 3 days after co-culture initiation by means of BrDU incorporation assays (A), [³H]-thymidine incorporation assays (C–D) and quantification of the number of S100 positive cell division profiles (B). dSCs lost the expression of O1 (A, lower panels) and resumed proliferation when placed in co-culture with DRG neurons (A and C). dSCs growing in an axon-free environment failed to proliferate despite stimulation with soluble mitogens (D). Undifferentiated SCs were used as controls in all experiments.

FIGURE 5.

Stimulation with live or disrupted DRG neurons induces SC dedifferentiation. cAMP-differentiated SCs (A, dSCs) or undifferentiated SCs (B, SCs) were stimulated with live DRG neurons (re-plated as a single cell suspension onto the SC cultures) or a preparation of disrupted neurons in medium containing CPT-cAMP and BrDU. Detection of O1, BrDU and neurofilament (NF) was done 3 days after treatment. An enlarged section (a) is provided in A (bottom panels) to show the axonal engagement of proliferating (BrDU positive) SCs. The low levels of O1 expression in some of these cells (arrowhead) was interpreted as indication of dedifferentiation. In C, SCs and dSCs were treated for the indicated time points with disrupted neurons and the levels of MCM2 expression and ErbB3 phosphorylation (P-ErbB3) were determined by Western blot. Note that MCM2 expression was increased to similar levels regardless of the state of differentiation and CPT-cAMP stimulation. In D–E, dSCs were incubated for 3 days in medium containing (middle panel) or lacking (left panel) disrupted DRG neurons and CPT-cAMP. Cultures were analyzed by immunofluorescence microscopy (D, O1/BrDU co-staining) or Western blot (E, O1/ErbB3). As expected, removal of the cAMP stimulus (-cAMP) induces O1 loss (D–E) in the absence of concomitant proliferation (D).

FIGURE 6.

An activity of DRG neurons reduces the expression of myelin markers without increasing the expression of immature SC markers in the presence of CPT-cAMP. Undifferentiated and cAMP-differentiated SCs were treated with disrupted DRG neurons for 3 days, as indicated, prior to analysis by immunofluorescence microscopy (A–B) and Western blot (C). Representative images of cultures double-immunostained with BrDU (red) and periaxin (Prx, green) antibodies (A) are provided along with quantitative data (B). Western blot analysis was performed in parallel samples (C) using several markers known to discriminate between differentiated (left panels) and immature (right panels) SCs. Two concentrations (10 and 20 μ g) of disrupted neurons were assayed (referred to as 1x and 2x in the figure). Densitometric data (optical density) for the expression of myelin markers is presented in panel D to illustrate the relative, dose-dependent changes in each individual marker.

FIGURE 7.

An activity specifically expressed in DRG neurons reduces O1 expression in cAMP-differentiated and myelinating SCs. In A–B, cultures of cAMP-differentiated SCs (dSCs) were stimulated with an equal protein concentration (20 μ g) of disrupted cells obtained from: purified DRG neurons, purified spinal cord neurons, SCs, dSCs, and Thy-1.1 positive fibroblasts. Stimulation was carried out for 3 days in the presence of CPT-cAMP. Cultures were immunostained with O1 antibodies and analyzed by fluorescence microscopy. Images from representative cultures (A) and quantification of O1 expression (B) is provided. In C–D, heavily myelinated SC-neuron cultures were subjected to enzymatic dissociation and separation of myelinating SCs by magnetic-activated cell sorting. Populations enriched in O1 positive SCs were plated in 24-well dishes and treated for 2 days with disrupted DRG neurons (20 μ g) in the absence (left panels) or presence (right panels) of CPT-cAMP. Analysis and quantification of O1 expression was performed as described in A–B.

FIGURE 8.

Contact between the SC membrane and the DRG axolemma is required for SC dedifferentiation. **A–B.** Axon density dependency of O1 loss in axon-related SCs. Differentiated SCs (dSCs) were seeded onto DRG cultures consisting of increasing neuronal densities, either in the absence or presence of CPT-cAMP. Note that a compact axonal web induced more extensive SC dedifferentiation (i.e. reduction of O1 expression) than an open axonal web. **C.** Lack of a dedifferentiating activity in the conditioned medium from high density neuronal cultures. dSCs were treated with freshly prepared neuronal medium or the conditioned medium from 30,000 DRG neurons (Neuron CM) in the absence or presence of CPT-cAMP. **D.** Maintenance of a differentiated O1 positive state in axon-deprived SCs. dSCs-neuron cultures were established and analyzed as described in A with the exception that an area devoid of axons located in the periphery of the culture well was included in the analysis. Representative pictures of the periphery (axons present only in the bottom left corner of the image) and the center (within the thick axonal outgrowth) of the culture are shown to denote that dSCs located in axon-free areas preserved high levels of O1 expression. **E–F.** Lack of effect of increased SC density on O1 loss in axon-related SCs. dSCs were plated at low (30,000 cells) and high (100,000 cells) density onto live DRG neurons (SC-neuron) or a laminin substrate (dSC-only). Note that dSCs lose O1 expression, acquire an elongated shape and proliferate (arrowhead in E, lower panel) upon contact with axons. O1 expression was determined 3 days after treatment initiation in all experiments.

FIGURE 9.

The dedifferentiating activity of DRG axons is a membrane-bound protein. **A–C.** *Recovery of a dedifferentiating activity in a membrane-enriched fraction from DRG neurons.*

Homogenized DRG neurons were subjected to differential centrifugation to obtain membrane-enriched (DRG membranes) and soluble (cytosol) fractions which were then used to stimulate non-differentiated (SCs, C) and differentiated (dSCs, A–B) SCs, respectively.

D–F. *Sensitivity of the neuronal dedifferentiating activity to trypsin digestion.* SCs and dSCs were stimulated with control (no trypsin) or trypsin-digested (trypsin) DRG membranes in the absence or presence of CPT-cAMP and BrDU, as indicated. Representative images (A and D) and quantitative analysis (B and E) of O1/BrDU co-staining in cultures fixed 3 days after treatment initiation are provided. The mitogenic activity of all preparations and the effectiveness of trypsinization were confirmed via [³H]-thymidine incorporation assays (C and F).

FIGURE 10.

The axonal dedifferentiating activity is different from the mitogenic activity. **A–D.** *Lack of a dedifferentiating activity in membranes enriched in neuregulin-1.* cAMP-differentiated SCs (dSCs) were left untreated (control) or stimulated with an equal protein concentration (20 μg) of membranes isolated from DRG neurons (DRG membranes) or HEK293 overexpressing neuregulin-1 (NRG-1) Type I and III. The incorporation of $[^3\text{H}]$ -thymidine (C) and the activation of ErbB signaling (D, Western blots) in undifferentiated SCs was used to confirm the mitogenic activity of all membrane preparations. **E–G.** *Effect of tyrosine kinase antagonists on proliferation and dedifferentiation.* SCs and dSCs were stimulated with DRG membranes in the absence or presence of CPT-cAMP provided alone or together with the indicated pharmacological inhibitors. These inhibitors were chosen based on their demonstrated specificity for ErbB receptors (EGFR/ErbB2 inhibitor), Src (PP2) and tyrosine kinases in general (genistein). Note that inhibitors effectively blocked S-phase entry, as determined by the incorporation of BrdU in SCs (E and G) and dSCs (E–F), without preventing O1 loss in response to DRG membrane stimulation (E–F). The dose-dependent anti-mitogenic action of the inhibitors was determined by $[^3\text{H}]$ -thymidine incorporation assays in undifferentiated SCs. Inhibitors were used at 10 μM unless indicated in the figure.

FIGURE 11.

An activity of DRG neurons induces SC dedifferentiation without reducing the expression of Krox-20 or increasing the expression of c-Jun, GFAP or p75^{NGFR}. Differentiated SCs (dSCs) were stimulated with disrupted DRG neurons (A–B) or DRG membranes collected by centrifugation (C–E) in the presence of CPT-cAMP for 3 days. A condition in which CPT-cAMP was removed from the culture medium (- cAMP) was also included in the analysis to re-establish p75^{NGFR}, GFAP and c-Jun expression (A–B) and concomitantly reduce that of Krox-20 (A–C). The cultures were analyzed by immunofluorescence microscopy and Western blot using the markers indicated in each panel. Undifferentiated cells (SCs) were used as controls in panels B–D. In E, Krox-20 immunolabeling (green) was performed together with BrDU (red, left panels) and MCM2 (red, right panels). A subset of Krox-20 positive SCs labelled positive for nuclear BrDU and MCM2 (arrows) indicative of cell cycle re-entry.

FIGURE 12. Axon-induced dedifferentiation is independent of JNK and ERK activation. Differentiated SCs (dSCs) were treated with disrupted (A, upper panels, and B) or live DRG neurons (A, lower panels) in the absence or presence of 10 μ M SP600125 or U0126, as indicated. Experiments were carried out in the presence of CPT-cAMP with the exception of a condition in which CPT-cAMP was removed to initiate JNK-dependent dedifferentiation (-cAMP). The cultures were analyzed by immunofluorescence microscopy (A) and Western blot (B) 3 days after stimulation or co-culture initiation. In C, the incorporation of [3 H]-thymidine was determined in undifferentiated SCs stimulated with mitogens (neuregulin/FBS) to confirm the anti-mitogenic activity of the inhibitors used.