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ARTICLE *in* JOURNAL OF NEUROSCIENCE RESEARCH · NOVEMBER 2009

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Journal of Neuroscience Research 87:3356–3368 (2009)

Neural Cell Adhesion Molecule Stimulates Survival of Premyelinating Oligodendrocytes Via the Fibroblast Growth Factor Receptor

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Axonal signals are critical in promoting the survival and maturation of oligodendrocytes during myelination, with contact-dependent signals thought to play a key role. However, the exact nature of these signals remains unclear. Neural cell adhesion molecule (NCAM) is expressed by both axons and oligodendrocytes and is ideally localized to transduce signals from the axon. This study sought to investigate the influence of NCAM on premyelinating oligodendrocytes in vitro. Both a soluble molecule comprising the extracellular domain of NCAM and a peptide derived from the fibroblast growth factor receptor (FGFR) binding motif within the first fibronectin domain stimulated a dose-dependent increase in survival of premyelinating oligodendrocytes in vitro. The survival effect was blocked by a mitogen-activated protein kinase (MAPK) inhibitor and an FGFR inhibitor, suggesting that activation of MAPK signalling pathways following interaction with the FGFR is involved in the survival effect of NCAM. Furthermore, NCAM presented in a cellular monolayer induced an increase in radial process outgrowth of oligodendrocyte progenitor cells. These data suggest that NCAM may play a role in axon–oligodendrocyte signalling during myelination, leading to an increase in oligodendrocyte survival and process outgrowth following axonal contact.

Key words: oligodendrocyte; NCAM; myelination; cell signalling

Myelination in the central nervous system (CNS) requires migration, proliferation, and differentiation of progenitor cells, followed by axonal engagement and wrapping of oligodendrocyte processes around the axon (Sherman and Brophy, 2005). The establishment of axon–glial contact during development is critical for the onset of myelination (David et al., 1984; Barres et al., 1993; Burne et al., 1996), and several studies have indicated a role for axonal contact in promoting oligodendrocyte survival and process outgrowth (Fernandez et al., 2000; Colognato et al., 2002, 2004; Taveggia et al., 2008). However, the precise axonal signals involved remain to be fully characterized.

Cell adhesion molecules (CAMs) expressed on the surface of both oligodendrocytes and axons are candidate molecules for mediating contact-dependent axon–glial signalling. Several studies have indicated that substrate-bound CAMs, including myelin-associated glycoprotein (MAG), neural cell adhesion molecule (NCAM), and $\alpha 6\beta 1$ integrin are able to promote the survival of oligodendrocytes in vitro (Gard et al., 1996; Frost et al., 1999; Colognato et al., 2002), and NCAM, N-cadherin, and MAG were also shown to promote process outgrowth of oligodendrocytes when presented on glass coverslips (Payne and Lemmon, 1993; Gard et al., 1996).

NCAM is spliced from a single gene to produce three major isoforms with molecular weights of 120, 140, and 180 kDa, of which NCAM-120 is the predominant form expressed by mature oligodendrocytes (Bhat and Silberberg, 1986, 1988; Trotter et al., 1989). The extracellular domain of NCAM consists of five Ig domains, which mediate homophilic adhesion (Ranheim et al., 1996; Soroka et al., 2002; Johnson et al., 2004) and two fibronectin type III domains (FnIII). Early during development, the majority of NCAM in the CNS is posttranslationally modified to carry polysialic acid (PSA) on the fifth Ig domain, which acts as a negative regulator of cell–cell interactions and is increasingly down-regulated as development progresses (Edelman and Chuong,

The first two authors contributed equally to this work.

Contract grant sponsor: Medical Research Council (to A.L.P., A.L.N.).

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Received 25 May 2009; Revised 16 July 2009; Accepted 17 July 2009

Published online 8 September 2009 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.22248

1982; Rougon et al., 1982; Bartsch et al., 1990). Stripping of PSA from NCAM expressed on the axon is required for myelination to commence during development in both rodent brain (Charles et al., 2000; Fewou et al., 2007) and human brain (Jakovcevski et al., 2007). Removal of PSA from axons in vitro leads to premature myelination in axon–oligodendrocyte cocultures (Charles et al., 2000). It seems likely that NCAM would then become involved in cell–cell adhesion events and stimulate intracellular signalling pathways in the oligodendrocyte. However, the influence of NCAM on oligodendrocyte function following removal of sialic acid residues has not been fully investigated.

In neuronal cultures, homophilic NCAM–NCAM binding directly stimulates intracellular signalling pathways to promote survival and neurite outgrowth, which involves activation of the fibroblast growth factor receptor 1 (FGFR1) and Fyn and a rise in intracellular calcium (Doherty et al., 1990; Saffell et al., 1992; Niethammer et al., 2002). This signalling is stimulated by the interaction of two motifs, designated *FRM* and *FGL*, within the FnIII domains of NCAM with the FGFR (Williams et al., 1994a; Saffell et al., 1997; Anderson et al., 2005; Kiselyov et al., 2005). Small synthetic ligands of NCAM derived from the FRM and FGL motifs have recently been shown to mimic many of the cellular functions of NCAM. These peptides stimulate survival of several neuronal types (Neiiendam et al., 2004; Anderson et al., 2005; Skibo et al., 2005), promote memory formation in rodent models (Cambon et al., 2004; Secher et al., 2006), and are neuroprotective against amyloid beta toxicity and ischemic insult both in vitro and in vivo (Skibo et al., 2005; Klementiev et al., 2007, 2008).

We have previously provided preliminary data suggesting that peptides derived from the FRM motif can act in a similar way on oligodendrocytes (Anderson et al., 2005). In the present study, soluble NCAM, peptides derived from the FRM motif, and an NCAM-expressing monolayer were used to investigate the role of NCAM in promoting survival and process outgrowth of oligodendrocytes in vitro. Both full-length NCAM-Fc and the FRM peptide stimulated a dose-dependent increase in survival of premyelinating oligodendrocytes, which involved signalling through MAPK and the FGFR. Furthermore, NCAM presented as a cellular monolayer stimulated process outgrowth of OPCs. Together these results suggest that NCAM expressed on the axon may be a key signalling molecule influencing survival and process outgrowth of oligodendrocytes following axonal contact during myelination and therefore could also influence repair following demyelination.

MATERIALS AND METHODS

Isolation of Neonatal OPCs by Immunopanning

All reagents were purchased from Sigma-Aldrich (Poole, United Kingdom), unless otherwise indicated. Cerebral cortices from postnatal day 4 Sprague Dawley rats (Harlan, Indianapolis, IN) were enzymatically dissociated with 0.25% trypsin-EDTA to produce a single-cell suspension. Neonatal OPCs were isolated by immunopanning with an antibody to the O4 antigen as described previously (Wu et al., 2001). Specifically bound cells were removed from the panning plate and plated at a density of 2.1×10^4 cells/cm^2 on poly-D-lysine (10 μg/ml)-coated coverslips in Dulbecco's modified Eagle's medium (DMEM) + 10% fetal bovine serum (FBS; PAA, Yeovil, United Kingdom). Medium was changed after 1 hr to defined serum-free oligodendrocyte progenitor (OLP) medium consisting of DMEM with the addition of 5 μg/ml insulin, 100 μg/ml transferrin, 0.05% BSA, 20 nM sodium selenite, 2 mM N-acetyl-L-cysteine, 2 mM L-glutamine, 1 mM sodium pyruvate, 20 nM progesterone, 30 ng/ml triiodothyronine, 10 nM hydrocortisone, 100 U/ml penicillin, and 100 μg/ml streptomycin. For survival experiments, cells were grown in OLP medium without the addition of insulin.

Growth Factors, Inhibitors, and NCAM-Derived Peptides

All NCAM peptides were synthesized by Mimotopes Ltd. (Melbourne, Australia). A dendrimeric peptide derived from the FRM region of NCAM consisted of four copies of the amino acid sequence DRVEPYSSTA linked at the C-terminus to a triple lysine backbone (FRM-d). A dendrimeric peptide with the reversed FRM amino acid sequence was used as a control. A cyclized version of the same sequence from the FRM motif joined by two cysteine residues (FRM-c) and a scrambled peptide with the same amino acids but in a random order, as a control, were also used. Peptides were used at concentrations of 5–100 μg/ml (3.6–72.9 nM). An NCAM-Fc fusion protein consisting of the extracellular domain of NCAM fused to the Fc region of human IgG_1 was produced using the pIg1 vector and a mammalian cell expression system (as described previously; Meiri et al., 1998). NCAM-Fc was used at a concentration of 5–50 μg/ml (33.8–338 nM). Recombinant human basic fibroblast growth factor (FGF-2; 0.01–1 ng/ml) and porcine platelet-derived growth factor-AA (PDGF; 0.1 ng/ml) were purchased from R&D Systems (Minneapolis, MN). PD173074, an FGF receptor inhibitor (50–100 nM); LY294002, a selective inhibitor of PI3 kinase (2–10 μM); and PD98059, an MAPK inhibitor (10–50 μM; all Calbiochem, San Diego, CA), were suspended in DMSO. DMSO final concentration did not exceed 0.1%, and an equivalent DMSO concentration was added to control cultures. All peptides, growth factors, and inhibitors were added 1 hr after plating. Control cells were grown in OLP medium with and without insulin (5 μg/ml) as positive and negative controls, respectively.

Immunocytochemistry

Primary antibodies used for immunocytochemistry were mouse anti-myelin basic protein (MBP; Boehringer Mannheim, Germany), rabbit anti-NCAM (Millipore, United Kingdom), mouse anti-myelin oligodendrocyte glycoprotein (MOG; Dr. S. Piddlesden, Cardiff, United Kingdom), and mouse O4 and O1 (obtained from the European Cell Culture Collection). Secondary antibodies used were goat anti-mouse

IgM-FITC (Sigma-Aldrich), biotinylated goat anti-mouse IgG, biotinylated goat anti-rabbit IgG, goat anti-rabbit IgG-Cy3 (all Stratech Scientific, Soham, United Kingdom), and streptavidin-conjugated Alexa Fluor 546 (Invitrogen, Paisley, United Kingdom). Cells were fixed for 20 min in 4% paraformaldehyde, blocked for 1 hr in 3% goat serum in PBS with 0.1% Triton X-100, incubated with primary antibodies for 45 min at room temperature (RT), washed in PBS, and incubated with secondary antibodies for 30 min at RT. After washing in PBS, coverslips were mounted in Vectashield (Vector Laboratories, Peterborough, United Kingdom).

Propidium Iodide Survival Assay

Survival assays were performed 4 days after addition of growth factors or NCAM peptides. Live cells were labelled with O4 antibody (1:5 in MEM-HEPES, 20 min on ice) and goat anti-mouse IgM-FITC (1:100, 15 min). After incubation with propidium iodide (Molecular Probes, Eugene, OR; 30 μM in PBS 5 min), cells were fixed and mounted as described above. All experiments were repeated at least three times, and individual conditions were performed in triplicate.

BrdU Incorporation

5-Bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich; 50 μM in OLP medium) was added to cultures 24 hr after plating and immunocytochemistry for O4 and BrdU carried out 16 hr later. Cells were labelled with O4-FITC and fixed with 4% PFA as described above. Coverslips were permeabilized for 5 min with 0.25% Triton X-100 (Sigma-Aldrich) and incubated with 2 M hydrochloric acid for 10 min to denature DNA, washed once with DMEM, and acid neutralized with 0.1 M sodium tetraborate, pH 8.5. Cultures were then incubated for 45 min with monoclonal anti-BrdU antibody (1:200; Chemicon, Temecula, CA) followed by goat anti-mouse IgG_1-Texas red secondary antibody (1:100; Cambridge Bioscience, Cambridge, United Kingdom) for 30 min and mounted as described above.

Western Blotting

OPCs were lysed on ice in RIPA buffer (50mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) containing protease inhibitor cocktail (Roche, Welwyn, United Kingdom) and phosphatase inhibitor cocktails (Sigma-Aldrich). Lysates were cleared by centrifugation and supernatants resuspended in 4× sample buffer (Invitrogen) for SDS-PAGE separation using Nupage Novex Bis-Tris gel system (Invitrogen) and transferred to a nitrocellulose membrane for immunoblotting. NCAM expression was detected with H300 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Analyses of cell signalling pathways were performed in OPCs that had been deprived of growth factors overnight, followed by a 15-min stimulation with insulin, FRM peptide, or NCAM-Fc, all at 5 μg/ml. Antiphospho-Akt (Ser473) was used to detect activated Akt, followed by stripping and reprobing with anti-Akt (both Cell Signaling Technology, NEB, Hitchin, United Kingdom). Antiphospho-ERK1/2 (Thr202/Tyr204) was used to detect activated ERK, and blots were reprobed with anti-ERK (both Cell Signalling Technology). As a protein loading control, anti-actin was used (Millipore). Peroxidase-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies (Dako Cytomaton, Ely, United Kingdom) were used and detected with an ECL Plus detection kit (GE Healthcare, Amersham, United Kingdom).

Coculture of OPCs With NCAM-Transfected Fibroblasts

Mouse NIH 3T3 fibroblasts constitutively expressing the human 140-kDa transmembrane isoform of NCAM at the cell surface (Doherty et al., 1989) or control untransfected 3T3 fibroblasts were maintained at 37°C and 8% CO_2 in DMEM (Invitrogen) supplemented with 10% FBS (PAA). Fibroblasts were plated at 5×10^4 cells/well in eight-chamber slides (LabTek; Nunc, Roskilde, Denmark) coated sequentially with poly-D-lysine and fibronectin and allowed to form confluent monolayers overnight. Twenty-four hours later, OPCs were isolated from neonatal rats by immunopanning as described above and plated on top of the monolayers at a density of 2.5×10^4 cells/ml in 400 μl/well OLP medium containing insulin.

Image Analysis

Stained cells were examined with a Nikon Eclipse 1000 fluorescence microscope, and digital images were captured with a QICAM Fast 1394 digital camera (Media Cybernetics, Marlow, United Kingdom). Composite images were produced in Image Pro Plus 5.1 (Media Cybernetics). The number of $O4^+PI^+$ or $O4^+BrdU^+$ double-labelled cells per ×20 objective field was counted manually and expressed as a percentage of the total number of $O4^+$ cells per field to quantify survival or proliferation, respectively. Twenty random fields were counted on three separate coverslips per test condition and from at least three independent experiments.

Quantification of Process Outgrowth

Images of 100 O4-immunostained cells per test condition were captured in Image Pro Plus, and the number of primary processes per cell was counted manually. The length of all processes per cell was measured using the manual trace tool. Coverslips were analyzed in triplicate, and the mean process length was calculated for each condition. Total cell area for the same 100 cells was also measured in Image Pro Plus 5.1.

Statistical Analysis

Significant differences between groups were analyzed by unpaired Student's *t*-test in Prism 4 software (GraphPad Software Inc., San Diego, CA), and $P < 0.05$ was considered significant.

RESULTS

Neonatal OPCs and Premyelinating Oligodendrocytes Express NCAM In Vitro

To investigate the role of CAMs in oligodendrocyte survival, OPCs were isolated at the O4-positive stage and allowed to differentiate into premyelinating oligodendrocytes, the point at which they would be contacting axons in vivo. Immunocytochemistry demonstrated sequential expression of myelin proteins and

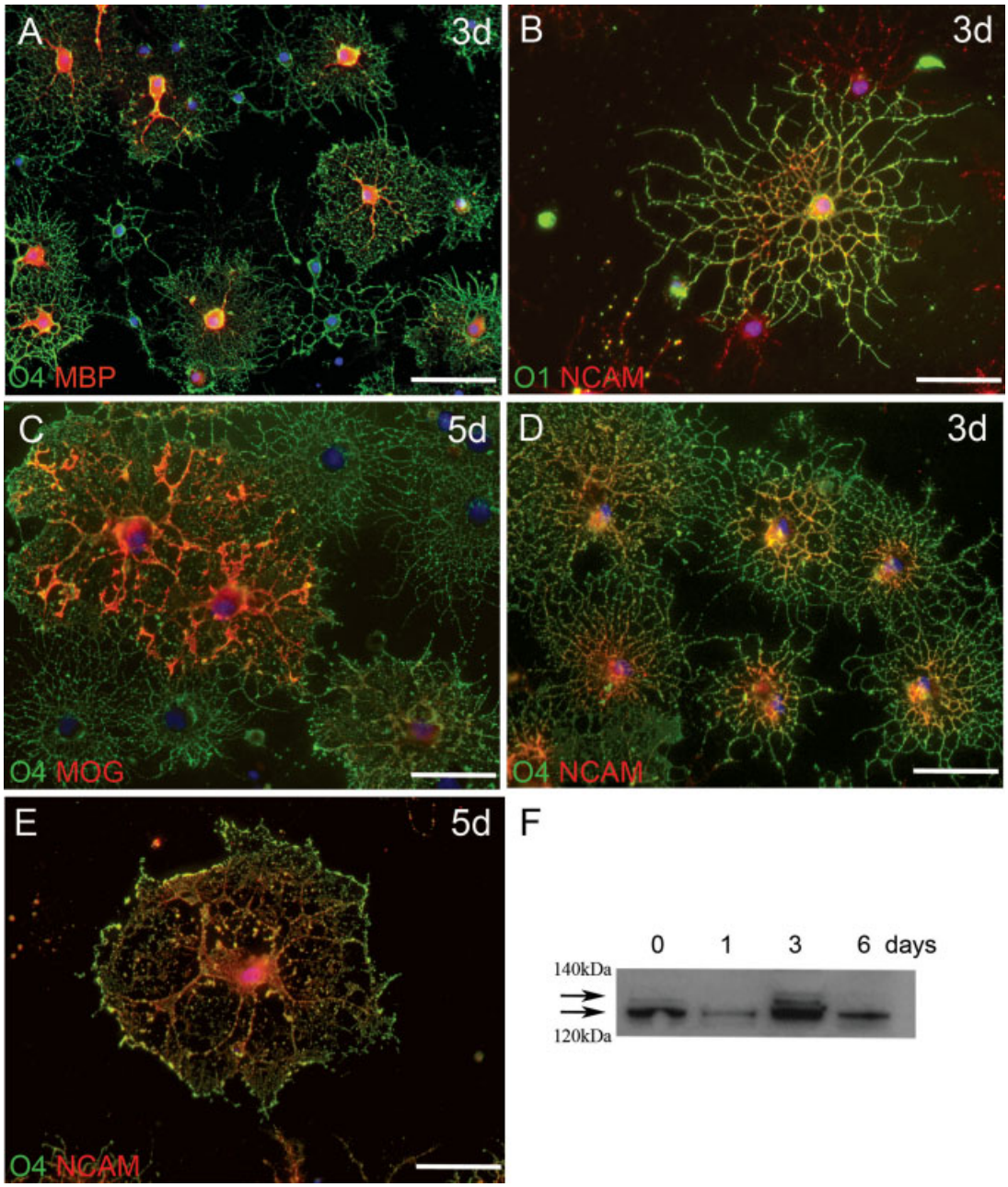
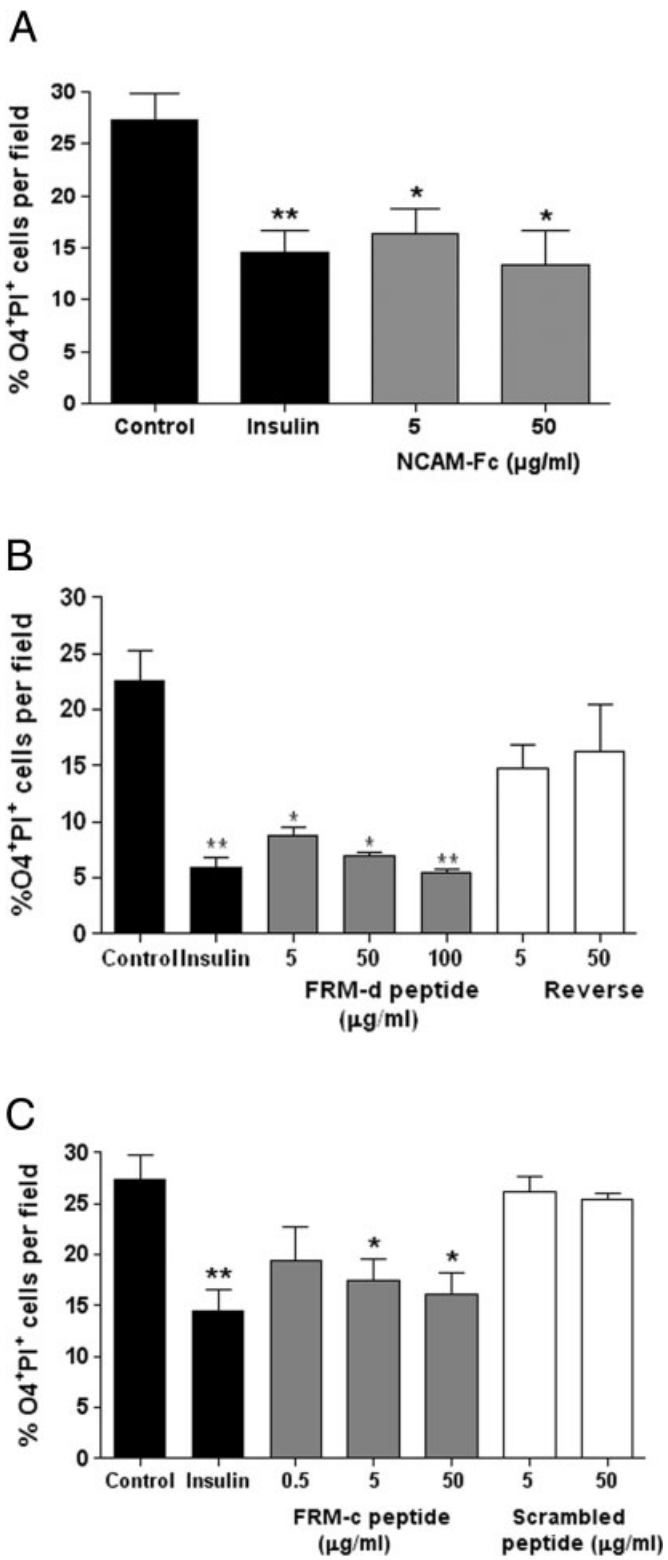


Fig. 1. NCAM is expressed by premyelinating oligodendrocytes in vitro. Premyelinating oligodendrocytes isolated from postnatal day 4 rats by O4 immunopanning were cultured for up to 5 days and labelled with the oligodendrocyte markers O4 or O1 (green). Oligodendrocytes expressed MBP (red in **A**) and galactocerebroside (O1, **B**) after 3 days and the late myelin marker MOG (red in **C**) after 5 days in vitro. Three days after isolation, oligodendrocytes also expressed NCAM (red in B,**D**) on the cell body and within the processes, whereas after 5 days oligodendrocytes no longer had defined processes and NCAM (red in **E**) was expressed throughout the myelin-like membrane. Western blotting on OPC cell lysates confirmed the expression of NCAM-120 and -140 in cells differentiated for 0–6 days (**F**). Scale bars = 50 μm.

lipids, with CNP expressed at 1–2 days, followed by MBP (Fig. 1A) and galactocerebroside (O1) at 2–3 days (Fig. 1B) and then MOG at later time points (4–5 days, Fig. 1C). After 5 days in culture, some cells had lost the defined processes of immature oligodendrocytes and had begun to produce rafts of myelin-like membrane (Fig. 1C). Expression of late-stage myelin proteins and the early formation of myelin sheaths suggest that these cells are equivalent to premyelinating oligodendrocytes seen in vivo (Hardy and Friedrich, 1996).

Immunocytochemistry showed that immature OPCs initially expressed low levels of NCAM, primarily

on the cell body. NCAM expression was greatly increased as OPCs matured and was present on the cell body and within the process networks after 3 days (Fig. 1B,D). After 5 days in vitro, many cells no longer had defined processes, and NCAM was expressed throughout the myelin-like membrane (Fig. 1E). Western blotting showed that OPCs that had been differentiated for 0–6 days in vitro predominantly expressed increasing levels of the 120-kDa NCAM isoform and to a lesser extent the 140-kDa isoform at 3 days (Fig. 1F). Thus, NCAM expression increases as OPCs differentiate and is highly expressed at the time point coincident with axonal contact in vivo.



NCAM-Fc and FRM Peptide Promote Survival of Oligodendrocytes

To investigate the effect of soluble NCAM on the survival of OPCs, a fusion protein consisting of the full extracellular domain of NCAM fused to the Fc region of human IgG_1 was tested for an effect on oligodendrocyte survival. Insulin (5 μg/ml), a well-characterized oligodendrocyte survival factor (Barres et al., 1992), was used as a positive control and induced an 11.6% increase in cell survival (Fig. 2A). As a negative control, insulin was removed from the medium, which resulted in significant cell death (Fig. 2A). All proteins/peptides were added to medium without insulin, and cell survival was assayed 4 days later. NCAM-Fc at 5 and 50 μg/ml induced an 11.0% and 14.0% significant increase in oligodendrocyte survival, as assessed by a decrease in the number of PI^+ cells compared with medium without insulin (50 μg/ml: 13.4% ± 3.3% PI^+ cells compared with 27.4% ± 2.5% without insulin; Fig. 2A).

To narrow the region of activity within the NCAM molecule responsible for the survival effect, a 10-amino-acid peptide (sequence DRVEPYSSTA) corresponding to the FRM region of NCAM was tested for its effects on cell survival. The FRM-d peptide (5, 50, and 100 μg/ml; Fig. 2B) and FRM-c peptide (0.5, 5, and 50 μg/ml; Fig. 2C) induced a dose-dependent reduction in the percentage of $O4^+PI^+$ cells per field, similar to that seen for NCAM-Fc. The highest concentration tested (100 μg/ml) produced approximately the same increase in survival as was seen with insulin (Fig. 2B). Peptides with a reversed

Fig. 2. Soluble NCAM and NCAM-derived FRM peptide increase survival of premyelinating oligodendrocytes in vitro. Oligodendrocyte survival was measured using propidium iodide (PI) labelling of pyknotic nuclei 4 days after addition of soluble NCAM-Fc consisting of the extracellular domain of NCAM. The number of $O4^+PI^+$ cells per field was counted and expressed as a percentage of the total $O4^+$ cells per field. NCAM-Fc stimulated a significant increase in oligodendrocyte survival, as measured by a decrease in PI^+ cells, at 5 and 50 μg/ml (**A**). The 10-amino-acid FRM peptides FRM-d (**B**) and FRM-c (**C**) also induced a dose-dependent increase in survival at 5, 50, and 100 μg/ml, whereas reverse and scrambled sequences of this peptide had no effect. Control cells were grown in medium with no growth factors or peptides, as a negative control, and insulin (5 μg/ml) was used as a positive control. Results are shown as mean ± SEM of four or five independent experiments, with each experiment analyzed in triplicate. Asterisks indicate a significant difference ($*P < 0.05$, $**P < 0.01$) compared with medium without insulin, assessed by unpaired Student's *t*-test.

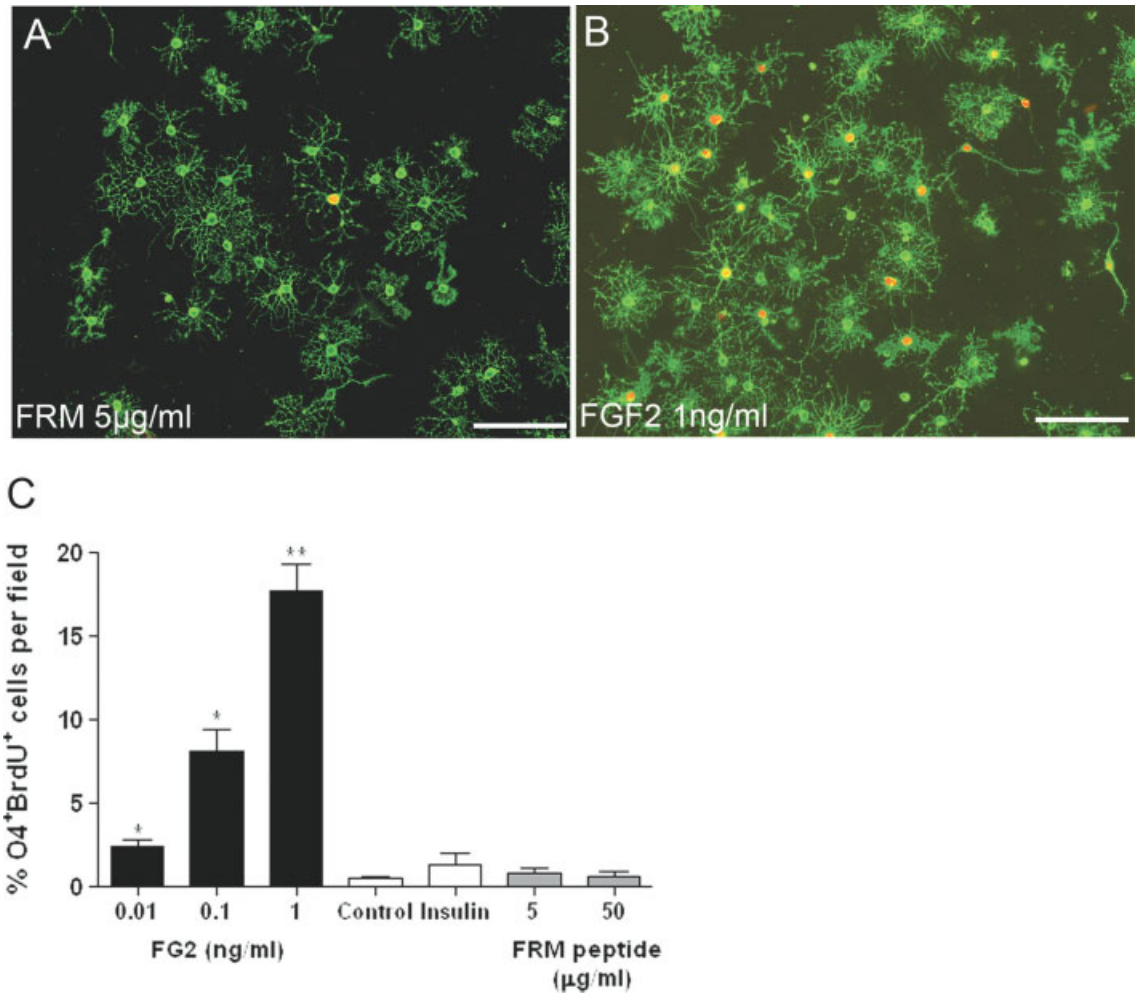


Fig. 3. FRM peptide does not increase OPC proliferation. OPCs were incubated with FRM-d peptide (**A**) or FGF-2 (**B**). BrdU was added 24 hr after plating, and immunocytochemistry for O4 and BrdU was carried out 16 hr later. The number of $O4^+BrdU^+$ cells per field was counted and expressed as a percentage of the total $O4^+$ cells per field (**C**). FGF2 produced a dose-dependent increase in OPC proliferation, whereas FRM peptide and insulin did not increase proliferation. Results are shown as mean ± SEM of three or four independent experiments. Three coverslips were analyzed per experiment. Asterisks indicate a significant difference ($\star P < 0.05$, $\star\star P < 0.01$) compared with control cells, assessed by unpaired Student's *t*-test. Scale bars = 100 µm.

or scrambled FRM sequence (5 and 50 µg/ml) had no significant effect on cell survival (Fig. 2B,C), indicating that the survival effect mediated by the FRM peptide was specific to that peptide sequence.

FRM Peptide Does Not Stimulate Proliferation of $O4^+$ Preoligodendrocytes

To test whether the FRM peptide stimulated proliferation of oligodendrocyte precursors, in addition to its survival effects, incorporation of the thymidine analogue BrdU was measured in the presence or absence of peptide, using FGF-2 as a positive control mitogen (Fig. 3). The FRM peptide did not induce a significant proliferative response at concentrations of 5 µg/ml or 50 µg/ml (Fig. 3A,C), concentrations at which it induced a significant survival response. The percentage of $BrdU^+$ cells in the presence of the highest concentration of the peptide was similar to that seen with insulin (1.2% and 1.4%, respectively). In comparison, FGF-2 (0.01–1 ng/ml) induced a dose-dependent increase in the number of $BrdU^+$ cells, indicating a significant proliferative response (Fig. 3B,C). Even at the lowest FGF-2 concentration tested (0.01 ng/ml), the percentage of $BrdU^+$ cells was approximately twice that of insulin (3% compared with 1.4%) and the FRM peptide at 50 µg/ml (1.2%).

Survival Effect of the FRM Peptide and NCAM-Fc Is Dependent on the FGF Receptor

To investigate whether the increase in OPC survival induced by FRM peptide and NCAM-Fc was dependent on activation of the FGFR, similar to that seen in neuronal cultures and fibroblasts (Anderson et al.,

2005), the specific FGFR inhibitor PD173074 (50 nM) was used. PD173074 prevented the survival effect of FRM peptide (5 μg/ml), NCAM-Fc (5 μg/ml), and FGF2 (0.1 ng/ml), inducing a return to approximately control survival levels, suggesting that the FGFR is crucial in transducing the FRM/NCAM-Fc survival signal (Fig. 4A). The survival response of insulin was not affected by addition of PD173074. As a vehicle control, 0.1% DMSO was added to parallel cultures and did not have a significant effect on survival (Fig. 4A).

Survival Effect of the FRM Peptide and NCAM-Fc Is MAP Kinase but Not PI3 Kinase Dependent

To elucidate further the downstream signalling pathways involved in the NCAM survival response, two pathways that are commonly associated with cell survival were investigated. The MEK inhibitor PD98059 induced a significant reversal of the survival effect of the FRM peptide, NCAM-Fc, and insulin at 50 μM and of FRM peptide and insulin at 2 μM (Fig. 4B). Western blotting for phosphorylated ERK1/2, which is activated downstream of MEK, also supported an involvement of the MAPK/ERK signalling pathway in the survival effect of the FRM peptide and NCAM-Fc (Fig. 4C), with increased phospho-ERK1/2 detected following 15 min stimulation with FRM peptide or NCAM-Fc.

The PI3K inhibitor LY294002 (2–10 μM) significantly inhibited the survival response to insulin (5 μg/ml; Fig. 4D). However, there was only a small, non-significant effect on the survival response to the FRM peptide and NCAM-Fc, even at 10 μM (Fig. 4D). Western blotting with a phosphorylation-state-specific antibody to Akt, the protein downstream of PI3K, also showed that FRM and NCAM-Fc did not stimulate the PI3K pathway (Fig. 4E), in contrast to insulin.

Cellular NCAM, but Not FRM Peptide or NCAM-Fc, Promotes Process Outgrowth of OPCs

To investigate whether NCAM can influence the process outgrowth of premyelinating oligodendrocytes, as it does for neurons (Meiri et al., 1998), OPCs were grown on confluent monolayers of NCAM-140-transfected 3T3 fibroblasts or control untransfected fibroblasts, to present NCAM within a cellular substratum. NCAM expression could not be detected on untransfected fibroblasts (Fig. 5A), whereas transfected cells showed robust expression of NCAM on the cell surface (Fig. 5B). OPCs grown on untransfected monolayers showed little process outgrowth after 24 hr, with the majority of cells retaining an immature phenotype with

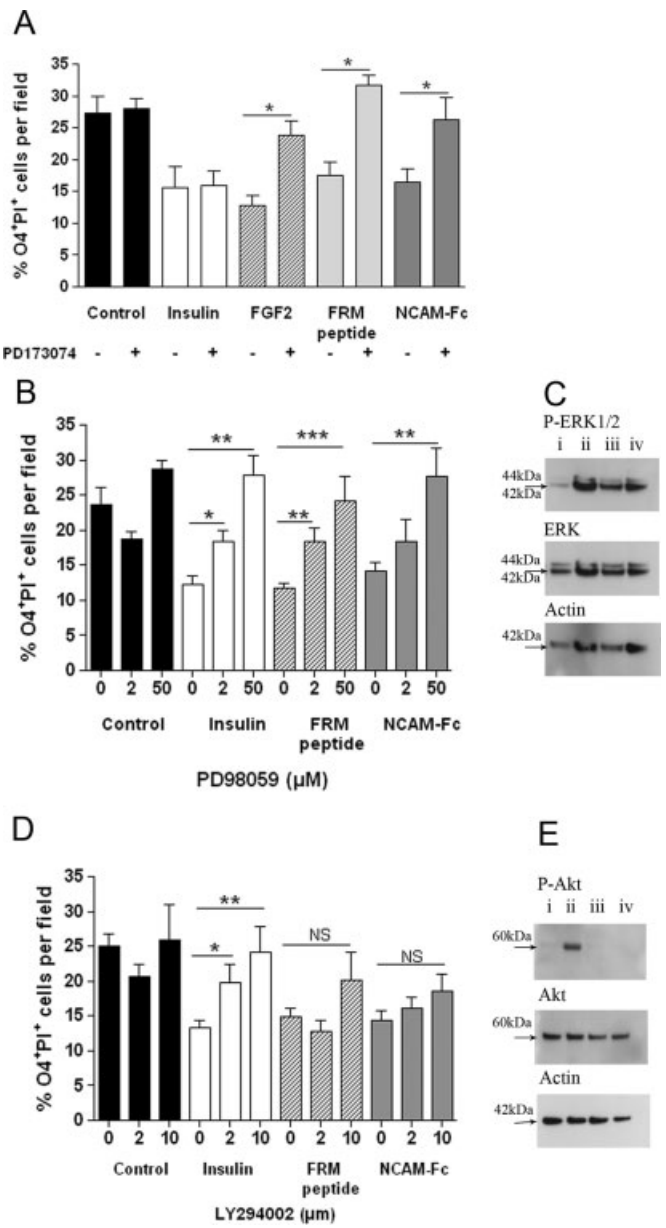


Fig. 4. NCAM stimulated survival is FGFR and MAPK dependent. OPCs were incubated with insulin (5 μg/ml), FGF2 (0.1 ng/ml), FRM-c peptide (5 μg/ml), or NCAM-Fc (5 μg/ml) in the presence or absence of inhibitors of the FGFR, MEK, and PI3K, and oligodendrocyte survival was measured by propidium iodide (PI) uptake 4 days later. The FGFR inhibitor PD173074 (50 nM; **A**) significantly blocked the survival effect of FGF2, FRM peptide, and NCAM-Fc. PD98059 (2–50 μM), a MEK inhibitor, significantly inhibited the survival effect of insulin, FRM peptide, and NCAM-Fc (**B**). LY294002 (2–10 μM), a PI3K inhibitor, blocked the survival effects of insulin but not FRM peptide or NCAM-Fc (**D**). Control cells were grown in medium without insulin as a negative control. Results are shown as mean ± SEM of at least three independent experiments, with each experiment analyzed in triplicate and assessed by unpaired Student's *t*-test. Asterisks indicate a significant difference between groups ($\star P < 0.05$, $\star\star P < 0.01$; NS, not significant). Immunoblot analysis of OPC cell lysates from untreated (i) or insulin (ii)-, FRM peptide (iii)-, or NCAM-Fc (iv)-treated cells (**C,E**) revealed an increase in phosphorylated ERK1/2 in OPCs stimulated by insulin, FRM peptide, and NCAM-Fc compared with control cells (C). Immunoblotting for phosphorylated Akt confirmed that only insulin activated this pathway (E). Images show representative blots of experiments repeated at least three times.

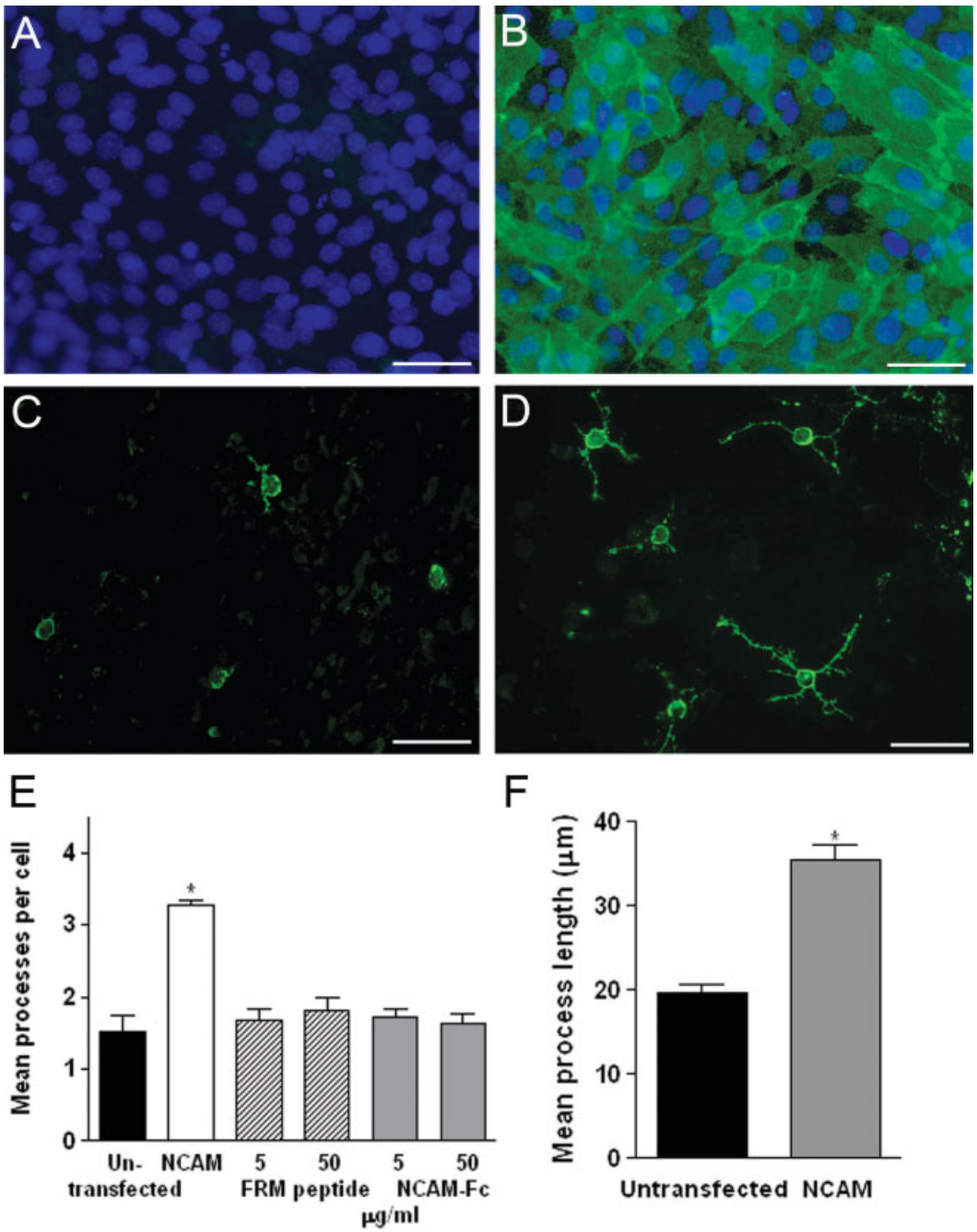


Fig. 5. Cellular NCAM stimulates OPC process outgrowth. Immunocytochemistry for NCAM demonstrated no expression of NCAM on control untransfected 3T3 fibroblasts (**A**) and robust expression of NCAM by fibroblasts stably transfected with NCAM-140 (**B**). OPCs were plated onto monolayers of untransfected or fibroblasts transfected with NCAM, in the presence or absence of FRM-c peptide (5 or 50 μg/ml) or NCAM-Fc (5 or 50 μg/ml). Process outgrowth was quantified after 1 day by using immunocytochemistry for O4. OPCs plated on NCAM-transfected fibroblasts (**D**) showed increased process outgrowth compared with those grown on untransfected cells (**C**). NCAM induced a significant increase in the number of primary processes per cell (**E**), but the addition of the FRM-c peptide (5 or 50 μg/ml) or NCAM-Fc (5 or 50 μg/ml) to OPCs grown on untransfected monolayers had no effect on process outgrowth (E). NCAM-transfected cells also increased the mean process length compared with untransfected cells (**F**). Results are shown as mean ± SEM of at least three independent experiments with a minimum of 150 cells analyzed per experiment. Asterisks indicate a significant difference ($^{*}P < 0.05$) compared with control, assessed by unpaired Student's *t*-test. Scale bars = 50 μm.

few processes (Fig. 5C). In contrast, OPCs grown on NCAM-transfected monolayers showed a significant increase in the number of processes per cell (Fig. 5D,E) and a marked increase in mean process length (approximately twofold) compared with OPCs grown on untransfected cells (Fig. 5F), suggesting that NCAM-140 is able to influence the process outgrowth of OPCs when presented on a cellular substratum. Addition of FRM peptide (5–50 μg/ml) or NCAM-Fc (5–50 μg/ml) to the media of OPCs grown on untransfected monolayers did not affect the process outgrowth compared with cells grown over untransfected monolayers in the absence of peptide/protein (Fig. 5E). In addition, when the process outgrowth of OPCs grown on coverslips for 24 hr was assessed, no significant difference between the mean process length on control (untreated) and FRM peptide (5–50 μg/ml)- or NCAM-Fc (5–50 μg/ml)-treated cells was observed (Fig. 6A). FRM peptide- and NCAM-Fc-stimulated cells typically had more processes per cell and fewer cells with zero or one process compared with control (Fig. 6B), indicating a possible slight effect on differentiation.

Cellular NCAM Increases OPC Cell Area but Does Not Influence OPC Proliferation or Myelin Protein Expression

To assess the ability of membrane-bound NCAM to influence differentiation of OPCs, the area covered by the process network and the percentage of cells expressing MBP was measured on each monolayer type after 48 hr. OPCs grown on NCAM monolayers displayed more extensive process networks and significantly increased cell area compared with OPCs grown on untransfected monolayers (Fig. 7A,B), indicating an increase in radial process outgrowth in response to NCAM (Fig. 7C). In comparison, neither FRM peptide (5 or 50 μg/ml) nor NCAM-Fc (5 or 50 μg/ml), when added to OPCs grown on coverslips, produced a significant change in cell area compared with untreated cells after 24 hr differentiation (Fig. 7E). MBP was expressed at high levels by a proportion of cells on both monolayers, with cells on NCAM monolayers showing increased amounts per cell resulting from an increase in cell surface area compared with OPCs on control monolayers. However, there was no significant difference in the percentage of MBP-positive cells among the different monolayers (Fig. 7A,B,D), suggesting that NCAM influences OPC process outgrowth independently of an effect on myelin protein expression.

Incorporation of the thymidine analogue BrdU was used to assess proliferation of OPCs plated on NCAM monolayers. No $BrdU^+$ OPCs were seen on control 3T3 monolayers, demonstrating that untransfected 3T3 fibroblasts did not inhibit process outgrowth by stimulating proliferation (data not shown). Very small numbers of $BrdU^+$ cells (0.7% ± 0.7) were observed on NCAM monolayers, but this was not statistically different from untransfected cells. Therefore, cellular NCAM was able to stimulate an increase in OPC process outgrowth in the absence of any effect on proliferation.

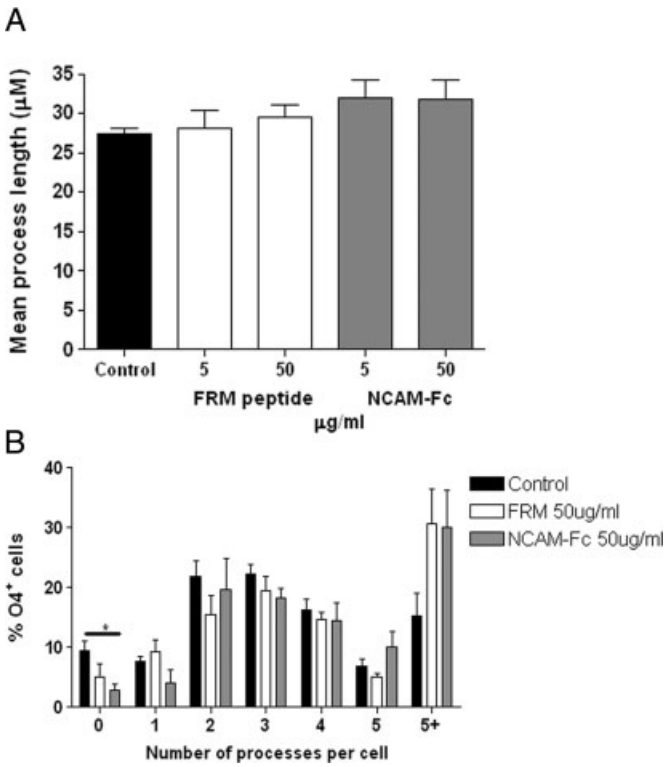


Fig. 6. Effect of FRM peptide and NCAM-Fc on OPC process outgrowth. OPCs were incubated with or without FRM-c peptide (5 or 50 μg/ml) or NCAM-Fc (5 or 50 μg/ml), and the extent of process outgrowth was quantified after 24 hr by using immunocytochemistry for O4. FRM-c peptide and NCAM-Fc did not significantly affect the mean process length (**A**) but did increase the number of processes per cell (**B**). Results are shown as mean ± SEM of at least three independent experiments, with a minimum of 150 cells analyzed per experiment.

DISCUSSION

The correct molecular interactions for the association of oligodendrocyte processes with the axonal membrane are essential not only for the process of myelination itself but also for the survival and initial process outgrowth of oligodendrocytes (Fernandez et al., 2000; Colognato et al., 2002). Here we present data suggesting that the cell adhesion molecule NCAM stimulates intracellular signalling pathways in oligodendrocytes, leading to an increase in survival. Soluble NCAM and peptides corresponding to the FRM motif of NCAM induced an increase in the survival of premyelinating oligodendrocytes, which could be blocked by both MEK kinase and FGFR inhibitors, implicating FGFR activation and MAPK signalling in the survival response induced by NCAM. Furthermore, NCAM, expressed on a cellular substratum, stimulated a significant increase in OPC process outgrowth. Together these data suggest an important role for NCAM in axon–oligodendrocyte signalling

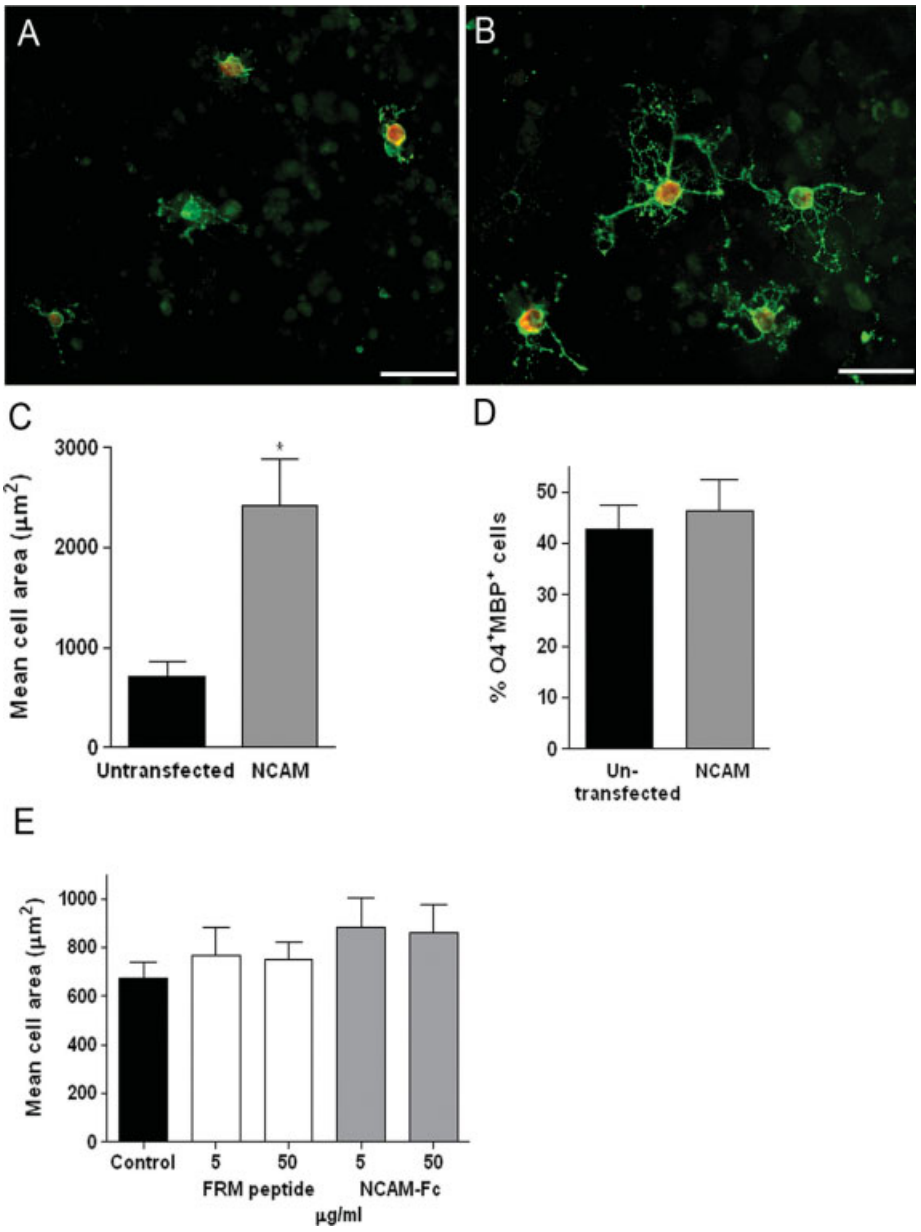


Fig. 7. Cellular NCAM influences process outgrowth of OPCs but not myelin protein expression. OPCs were plated onto monolayers of untransfected fibroblasts (**A**) or NCAM-140-transfected fibroblasts (**B**) and immunocytochemistry for O4 (green) and MBP (red) performed after 2 days. NCAM induced a significant increase in cell area (B) compared with control (A,**C**). NCAM had no effect on the number of MBP-positive OPCs compared with control (A,B,**D**). OPC cell area was measured on cells grown in the absence of a fibroblast monolayer after 24 hr, in the presence of FRM peptide (5 or 50 μg/ml) or NCAM-Fc (5 or 50 μg/ml; **E**). FRM-c peptide and NCAM-Fc did not significantly affect cell area compared with control cells (E). Results are shown as mean ± SEM of at least three independent experiments, and at least 150 cells were analyzed per experiment. Asterisks indicate a significant difference ($\star P < 0.05$) compared with control monolayers, as assessed by unpaired Student's *t*-test. Scale bars = 50 μm.

during development, in addition to its known role in adhesion.

Soluble NCAM Enhances Oligodendrocyte Survival Via the FGFR and MAPK Signalling Pathways

Homophilic binding of axon–oligodendrocyte NCAM during the initial stages of myelination is a candidate mechanism for transducing signals between the axon and the oligodendrocyte. In the present study, both the extracellular domain of NCAM and peptides derived from the FRM motif induced a dose-dependent increase in oligodendrocyte survival, which agrees with previous findings showing the survival-promoting properties of NCAM and other CAMs on oligodendrocytes in culture (Gard et al., 1996). These results suggest that the FRM site is critical for the survival effect of NCAM. The FRM and FGL sites, located within the first and second FnIII domains of NCAM, respectively, are hypothesized to form a site that is able to interact with FGFR1 in neurons and stimulate intracellular signalling to increase survival and neurite outgrowth (Doherty et al., 1990; Williams et al., 1994a,b; Kiselyov et al., 2003; Neiiendam et al., 2004; Anderson et al., 2005; Carafoli et al., 2008), indicating a general role for CAM signalling in cell survival. We have shown here that the survival effects of FRM peptide and NCAM-Fc on oligodendrocytes are also dependent on the FGFR, using a small molecule inhibitor that can specifically inhibit all

FGFRs (Bansal et al., 2003). This survival effect was observed in the absence of exogenous FGF2, suggesting that FRM peptide and NCAM-Fc do not require the presence of FGF2 to elicit their effects. However, a low level of FGF2 release by the cells in the culture system cannot be excluded. The expression of the different FGFRs on OPCs and oligodendrocytes has been previously mapped out (Bansal et al., 1996), and, although FGFR1 has been shown to be the important subtype in controlling the effects of FGF2 on OPC differentiation (Zhou et al., 2006), it is possible that FRM peptide and NCAM-Fc may act via any of these receptor subtypes.

The survival effect of the FGL peptide on neurons could be blocked by inhibitors to FGFR, MEK, and PI3K (Neiiendam et al., 2004), but the signalling pathways associated with the effect of FRM peptides have yet to be assessed. In this study, the survival response of the FRM peptide and NCAM-Fc was shown to be dependent on the MAPK/ERK pathway but not the PI3 kinase pathway. Sustained activation of the MAPK pathway is associated with increased cell survival, and, although activation of the PI3K pathway is important for NCAM-mediated cell survival in neurons, a developmental switch is reported to take place in oligodendrocytes, so that survival signals are initially dependent on PI3K but later switch to MAPK (Colognato et al., 2002). It is likely that, at the maturation stage investigated here, the point at which contact-dependent signals take over from soluble growth factors, oligodendrocyte-lineage cells are dependent on the MAPK pathway. This also suggests that different pathways are activated by NCAM in oligodendrocytes compared with neurons.

Cellular NCAM, but Not Soluble NCAM, Stimulates Process Outgrowth of OPCs but Does Not Influence Proliferation or Myelin Protein Expression

The role of NCAM in promoting oligodendrocyte process outgrowth was examined using the well-characterized transfected fibroblast coculture system (Doherty et al., 1989, 1990). OPCs grown on NCAM-transfected monolayers showed a marked change in morphology, with a significant increase in the number of processes per cell, process length, and cell area, suggesting that NCAM is able to stimulate radial process outgrowth of OPCs. The expression level of NCAM within the transfected fibroblasts used in this study has been shown to be within the physiological range and approximates the interactions found in vivo (Doherty et al., 1990, 1991). The increase in OPC process outgrowth in response to cellular NCAM expression is also in agreement with studies showing that NCAM stimulates process outgrowth in primary neurons. Soluble versions of NCAM were unable to stimulate an increase in OPC process outgrowth, whereas FRM peptide (Anderson et al., 2005) and NCAM-Fc (Meiri et al., 1998) both increased neurite outgrowth. It is possible that oligodendrocytes require NCAM to be expressed in a cellular substratum so that it is in the correct orientation. This suggests that different signalling pathways may be required to stimulate process outgrowth compared with survival in OPCs or that activation of signalling may not be sufficient to induce process outgrowth and CAM–CAM interactions in trans may also be required.

An Important Role for NCAM in Oligodendrocyte Development and Myelination

During development, the presence of PSA-NCAM on axons inhibits myelination, and the removal of PSA is an important regulator of the onset of myelination (Charles et al., 2000), allowing oligodendrocyte processes to attach to axons. Together with our present study, these data support a putative inhibitory role for PSA-NCAM in myelination (Charles et al., 2000, 2002) and a stimulatory role for nonpolysialylated NCAM. Axon–oligodendrocyte interactions, involving NCAM–NCAM homophilic binding or other heterophilic CAM interactions, may lead to activation of oligodendrocyte survival and process outgrowth via activation of intracellular signalling. Premyelinating oligodendrocytes that do not contact axons and receive the correct survival signals will die by apoptosis (Barres et al., 1993; Trapp et al., 1997). Thus, NCAM/FGFR signalling may contribute to the target-dependent survival of oligodendrocytes, most likely in conjunction with other signalling pathways (Baron et al., 2005).

Expression of adhesion molecules on axons is likely to be important for the reestablishment of axoglial interactions following demyelination, and, if CAMs also promote process outgrowth and survival of oligodendrocytes, then they may be important in promoting remyelination and repair in the CNS. PSA-NCAM was recently shown to be up-regulated on demyelinated but not remyelinated axons within MS lesions (Charles et al., 2002), suggesting that PSA-NCAM may also be inhibitory to remyelination. Loss of NCAM expression or a change to PSA-NCAM within lesions may limit the capacity for repair by blocking the reattachment of oligodendrocytes to axons and inhibiting the survival and process outgrowth of adult OPCs. Therefore, changes in axonal CAM expression may determine the success of remyelination, even in the presence of competent oligodendrocytes.

In conclusion, this study highlights the possible role of CAMs as contact-dependent mediators of signalling cues to oligodendrocytes during myelination. Axonal NCAM, or other adhesion molecules, may interact in trans with oligodendrocyte NCAM during the initial stages of myelination in vivo. This interaction may lead to an FRM-mediated cis interaction between NCAM and the FGFR in the oligodendrocyte membrane and subsequent activation of signalling pathways leading to increased survival. NCAM–FGFR interaction, leading to MAPK activation, represents a novel signalling system not previously explored in oligodendrocytes and is likely to act in concert with other factors to influence oligodendrocyte development. These data also further highlight the

importance of the FRM domain in the survival bioactivity of NCAM. Several other CAMs, such as N-cadherin and L1, also contain FRM motifs within their FnIII domains (Williams et al., 1994a), and their interaction, either directly or indirectly, with the FGFR in vivo may also lead to an increase in oligodendrocyte survival. This study also demonstrates that a contact-dependent element is required for NCAM to increase process outgrowth, suggesting that the mechanisms of survival and outgrowth are different.

ACKNOWLEDGMENTS

We dedicate this work to Prof. Stephen Pfeiffer, who was both a mentor and friend to R.R. and was responsible for teaching us how to carry out immunopanning during a sabbatical at UCHC.

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