N-CAM Binding Inhibits the Proliferation of Hippocampal Progenitor Cells and Promotes Their Differentiation to a Neuronal Phenotype

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Cell adhesion molecules (CAMs) play important roles during the development of the nervous system. On the basis of our previous observations that binding of the neural CAM (N-CAM) inhibits astrocyte proliferation and alters gene expression, we hypothesized that N-CAM may influence the balance between the proliferation and the differentiation of neural progenitor cells. Rat and mouse hippocampal progenitor cells were cultured and showed dependence on basic FGF for proliferation, immunoreactivity for nestin, the presence of limited numbers of differentiated cells, and the ability to generate glial cells and neurons under different culture conditions. Addition of soluble N-CAM reduced cell proliferation in a dose-dependent manner with no evidence of apoptosis. The inhibition of proliferation by N-CAM was accompanied by an induction of differentiation to the neuronal lineage, as indicated by a twofold increase in the percentage of microtubule-associated protein 2-positive cells even in the presence of mitogenic growth factors. Experiments using hippocampal cells from N-CAM knock-out mice indicated that N-CAM on the cell surface is not required for these effects, suggesting the existence of heterophilic signaling. These results support a role for N-CAM and N-CAM ligands in the inhibition of proliferation and the induction of neural differentiation of hippocampal neural progenitor cells.

Key words: neural stem cells; progenitors; neural cell adhesion molecule (N-CAM); inhibition of proliferation; neuronal differentiation; N-CAM knock-out; N-CAM heterophile

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stimulation of N-CAM neuronal differentiation

MATERIALS AND METHODS

Hippocampal progenitor cell culture. Hippocampi were dissected from embryonic day 17 (E17) to E18 rat embryos or E16 to E17 mouse embryos into HBSS without calcium or magnesium and containing penicillin (50 U/ml), streptomycin (100 μg/ml), and glutamine (2 mM) (Life Technologies, Gaithersburg, MD). The tissue was collected by centrifugation for 2 min at 500 × g, and the buffer was removed. To dissociate the tissue, we incubated it for 20 min at 37°C in 0.1% trypsin, 1 mM EDTA (Life Technologies), and 0.1 mg/ml DNase I (Sigma, St. Louis, MO). A mixture of HBSS, BSA (0.1%), trypsin inhibitor (0.025 mg/ml; Sigma), and DNase I (0.1 mg/ml) was added before pelleting the tissue for 5 min at 300 × g. The dissociated tissue was incubated in this same mixture for 1 min and triturated with glass Pasteur pipettes of narrowing diameter. The resulting dissociated cells were centrifuged and resuspended in Neurobasal medium supplemented with penicillin, streptomycin, glutamine, and the synthetic mixture B27 (Life Technologies) (NB/B27), in the presence of 20 ng/ml basic FGF (bFGF; Sigma). The cells were plated on a poly-l-lysine and laminin substrate for proliferation as well as differentiation assays.

Preparation of N-CAM, extracellular N-CAM, and recombinant N-CAM domains. N-CAM was purified from early postnatal rat brains or chicken brains as described previously (Hoffman et al., 1982; Sporns et al., 1995) and was shown to contain the polysialic acid characteristic of N-CAM from young brain tissue. Extracellular N-CAM was obtained by digestion of chicken brain membranes with the Staphylococcus aureus V8 protease as described previously (Cunningham et al., 1983) and subsequently subjected to immunofinity purification using an antibody specific to the extracellular part of N-CAM. The various N-CAM recombinant proteins were produced in Escherichia coli and purified as reported previously (Ranheim et al., 1996).

Proliferation assay. Hippocampal cells were plated at a density of 20,000 cells/ml in eight-chamber glass slides coated with poly-l-lysine and laminin in NB/B27 medium containing 20 ng/ml bFGF for 2 d (0.25 ml per well). After 48 hr of treatment with N-CAM (10 μg/ml), the cells were washed with PBS, fixed with 4% PFA, and processed using the immunohistochemistry cell proliferation ELISA-BrdU kit (Boehringer Mannheim, Indianapolis, IN) according to the protocol provided by the manufacturer. In some experiments, cells were pretreated (1 hr) with heparin, heparinase, chondroitin sulfate, glycosidases [N-glycosidase endo F and O-glycosidase (Boehringer Mannheim)], or an anti-FGF receptor (Upstate Biotechnology, Lake Placid, NY).

Apoptosis assay. Hippocampal cells were plated at a density of 20,000 cells/ml on eight-chamber glass slides coated with poly-l-lysine and laminin in NB/B27 medium containing 20 ng/ml bFGF for 2 d (0.25 ml per well). After 48 hr of treatment with N-CAM (10 μg/ml), the cells were fixed with 4% PFA (Sigma). The TUNEL assay was used to assess apoptosis. Briefly, fragmented DNA was extended by the terminal deoxynucleotidyl transferase (250 U/ml; Boehringer Mannheim) that incorporated chromotid body-P14-DUMP (5 μM; Molecular Probes, Eugene, OR). To reveal nuclear DNA, the cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma) applied at 1 μg/ml for 2 min at room temperature. The nuclei of healthy cells (blue) and the nuclei of apoptotic cells (green) were counted using a fluorescence microscope.

Immunocytochemistry for BrdU, microtubule-associated protein 2, GFAP, and nestin. For all antibody staining, cells were fixed with 4% PFA. For BrdU immunostaining, fixed cells were treated for 5 min with 0.05% NaOH. Nonspecific binding sites were blocked with 5% goat serum in PBS, and the cells were permeabilized with 1% Triton X-100 (Sigma). All steps were performed at room temperature. Nestin monoclonal antibody (PharMingen, San Diego, CA) and microtubule-associated protein 2 (MAP2) monoclonal antibody (Sigma) were diluted 1:1000. BrdU monoclonal antibody (Dako, Carpinteria, CA) was diluted 1:20. For GFAP determination, polyclonal anti-GFAP (Dako) was applied at a dilution of 1:2000. MAP2 and GFAP antibodies were applied simultaneously. Primary antibodies were incubated with the cells for 1 hr. After washing three times for 10 min with 0.1% Triton X-100, the secondary antibodies [Texas Red-anti-mouse for MAP2 (1:500) and FITC-anti-rabbit for GFAP (1:500); both from Molecular Probes] were applied and incubated for 1 hr, and the slides were washed as described above. All antibodies were revealed using the biotin–avidin–horseradish peroxidase vectastain system (Vector Laboratories, Burlingame, CA) and DAB in tablets as a substrate (Sigma). BrdU- and nestin-positive cells were observed in bright field. For MAP2 and GFAP labeling, cell nuclei were counterstained with DAPI, and the microscope slides were mounted with Slowfade-mounting media (Molecular Probes) and observed by fluorescence microscopy.

Differentiation assay. Hippocampal cells were grown on poly-l-lysine- and laminin-coated eight-chamber glass slides (Becton Dickinson, Bedford, MA) in NB/B27 medium in the presence of bFGF (20 ng/ml), at a density of 5000 cells per well for 4 d. At that time, the medium was changed, and the cells were treated for 3 additional days with N-CAM or with various N-CAM fragments (BDNF, neurotrophin-3 (NT-3), PDGF, insulin-like growth factor (IGF-1), CNTF, or FCS in NB/B27 with bFGF) and at the concentrations indicated in the figure legends. As a control, BDNF was also added in the absence of bFGF to stimulate neuronal differentiation under the conditions reported previously (Vicario-Abejon et al., 1995). At the end of the seventh day, cells were fixed and processed for MAP2 and GFAP immunocytochemistry as described above. All the cells were counterstained with DAPI to reveal cell nuclei. For each treatment, five fields were counted for total cells (DAPI), neuronal cells (MAP2⁺), and astrocytes (GFAP⁺). The results of the five fields were averaged and corresponded to a minimum number of 200 cells. The percentage of MAP2⁺ or GFAP⁺ cells was then calculated.

Binding of N-CAM and N-CAM fragments in vitro to live progenitor cells from N-CAM knock-out mice. Hippocampal cells from N-CAM knock-out (KO) mouse embryos (Holst et al., 1998) were grown on poly-l-lysine and laminin substrate in glass multichamber slides (Becton Dickinson) for 1–2 d in NB/B27 medium in the presence of bFGF (20 ng/ml). They were treated with various N-CAM protein reagents for 2 hr at 37°C. The cells were washed with PBS, fixed with 4% PFA, and processed using immunocytochemistry for the binding of N-CAM and N-CAM fragments. A rabbit polyclonal antibody directed against the extracellular domain of N-CAM that recognizes both rat and chicken N-CAM (anti-N-CAM S27) was used at 3 μg/ml. Specific antibodies for each N-CAM domain were used at 10 μg/ml (Ranheim et al., 1996). The secondary antibody was an FITC-anti-rabbit antibody (Molecular Probes) (1:500). In some instances, cells were pretreated (1 hr) with heparin, heparinase, chondroitin sulfate, or glycosidases (N-glycosidase endo F and O-glycosidase) (all from Boehringer Mannheim).

RESULTS

Characterization of the rat hippocampal progenitor cell culture system

As described in the introductory remarks, several studies have provided culture conditions that support the growth and expansion of hippocampal neural progenitors. These conditions were adapted for the present study, and the resulting cultures were characterized. Four hours after plating rat hippocampal cells in bFGF-supplemented medium, some cells either formed “neurospheres” in the absence of a substrate that supported adhesion or formed colonies in the presence of a substrate that supported adhesion. After 4 d on a poly-l-lysine and laminin substrate, the cells grew in loose colonies of individually identifiable cells (Fig. 1A). At this time, ~70% of the cells incorporated BrdU over 24 hr (Fig. 1B). bFGF or a combination of both epidermal growth factor (EGF) and bFGF supported cell proliferation, whereas...
EGF alone was an effective but less potent mitogen (data not shown). The cells were unipolar or bipolar with short extensions (Fig. 1A), and 95% of the cells expressed nestin, an intermediate filament protein present in neural stem cells and progenitors (Lendhal et al., 1990) (Fig. 1C). These cells could be subcultured for up to 6 months in the presence of bFGF.

To stimulate differentiation, cells grown for 4 d in medium with bFGF were shifted to medium without bFGF and grown for an additional 2 d. Following the convention established in other studies (Ghosh and Greenberg, 1995; Arsenijevic and Weiss, 1998), cells of the neuronal lineage were identified by MAP2 expression, and cells of the astrocyte lineage were identified by GFAP expression. Immunostaining for MAP2 and GFAP revealed many cells expressing each differentiation marker (Fig. 1D; 25% GFAP<sup>+</sup> and 70% MAP2<sup>+</sup>). S100 antibodies stained all GFAP<sup>+</sup> cells and no others. Approximately 1% of the cells expressed the oligodendrocyte marker (data not shown). In the later-passage cells, cell phenotypes stimulated by growth factor removal were in approximately the same proportion as those in primary cultures. These observations suggest that the proliferating cells present in the original culture correspond to multipotential neural progenitor cells.

Inhibition of rat progenitor cell proliferation by N-CAM and an extracellular N-CAM fragment

We found previously that N-CAM inhibited astrocyte proliferation in vitro and in vivo after a lesion and that the third immunoglobulin domain of N-CAM (Ig III) was effective at reducing astrocyte proliferation (Krushel et al., 1995, 1998; Sporns et al., 1995). We therefore asked whether this effect occurred in other proliferative cells of the nervous system, including neural progenitor cells, which have been demonstrated to express N-CAM (Rousselot et al., 1995; Mayer-Proschel et al., 1997) (M.-C. Amoureux and K. L. Crossin, unpublished observations). Purified N-CAM inhibited neural progenitor cell proliferation stimulated by bFGF in a dose-dependent manner, reaching a maximum of 94% at a dose of 10 μg/ml (Fig. 2A). A concentration of N-CAM > 1.25 μg/ml was necessary to obtain measurable inhibition, and the IC<sub>50</sub> was determined to be ~1.5 μg/ml. Preabsorption of the N-CAM solution with beads coated with monoclonal antibody against N-CAM abolished the inhibitory activity (Fig. 2B), indicating that the effect was specifically caused by N-CAM. The inhibitory activity of N-CAM was also observed when N-CAM was coated onto the dish in addition to the poly-L-lysine and/or laminin substrate (data not shown). N-CAM (10 μg/ml) could also inhibit EGF-stimulated progenitor proliferation by 72 ± 16%, indicating that the effect of N-CAM is not attributable to a particular mitogen. Recombinant N-CAM Ig III fragment and an extracellular fragment of native N-CAM purified from V8 protease-treated brain membranes (Cunningham et al., 1983) were also tested in this assay (Fig. 2B). The native extracellular domain inhibited cell proliferation, whereas the recombinant Ig III domain did not. It was surprising that neither Ig III (Fig. 2B) nor an antibody against N-CAM (data not shown) inhibited proliferation, because both of these reagents were effective in inhibiting astrocyte proliferation (Sporns et al., 1995; Krushel et al., 1998). In the absence of bFGF, BrdU incorpora-
tion was very low after 2 d and was unaffected by N-CAM treatment (data not shown).

**N-CAM does not induce cell death**

A possible explanation for the decrease in BrdU incorporation is that N-CAM reagents induced cell death. Indeed, a previous study suggested that N-CAM cross-linking induced by the application of an N-CAM antibody resulted in apoptosis of cortical neurons in vitro (Azizeh et al., 1998). Apoptosis was measured by TUNEL labeling with fluorescent nucleotides with or without N-CAM treatment for 2 d (Fig. 3). The percentage of TUNEL-positive cells was measured as a fraction of the total cells revealed by the DAPI nuclear stain. Few cells underwent apoptosis in either condition: 5.4% (±4.6%) for untreated and 8.0% (±3.0%) for N-CAM-treated cultures. Moreover, no pyknotic nuclei were observed in either condition. Therefore the large decrease in BrdU incorporation in the presence of N-CAM could not be explained by an increase in cell death.

**N-CAM binding leads to increased progenitor cell differentiation**

Because proliferation was altered by N-CAM binding, we hypothesized that neural progenitor differentiation might also be affected. To address this possibility, we quantified the number of differentiated cells by immunocytochemistry using antibodies to MAP2 and GFAP (see Fig. 1D). bFGF was maintained in the medium (NB/B27) to preserve the conditions under which N-CAM affected proliferation. When N-CAM was added (10 μg/ml) to rat hippocampal progenitor cells, ~85% of the cells expressed MAP2, a 2.3 (±0.3)-fold (n = 7) increase over control cells. N-CAM treatment also resulted in a reduction in the number of GFAP-expressing cells from 12 to 2% of the total cells (Fig. 4A). This effect on differentiation could not be mimicked with any recombinant Ig N-CAM domains (Ig I–II, Ig III, Ig IV, or Ig V domains) applied at concentrations up to 50 μg/ml (data not shown).

To control for possible species differences in neural progenitors (Kempermann et al., 1998a) as well as to characterize a mouse progenitor culture that could be used with cells from genetically altered animals, we assessed the effect of N-CAM on the differentiation of mouse progenitor cells (Figs. 4B, 5). In medium supplemented with bFGF, many cells were MAP2⁺ (~40%), but in contrast to rat progenitor cells cultured under identical conditions, few mouse cells expressed GFAP (~12% in rat vs 0.33% in mouse) (Figs. 4B, 5). However, in agreement with the findings using rat cultures, N-CAM treatment significantly increased the percentage of MAP2⁺ cells (Figs. 4B, 5). Although N-CAM did...
not affect the percentage of astrocytes from mouse progenitor cell cultures (Fig. 5A), a GFAP 
positive population could be induced by the addition of CNTF or FCS (Figs. 4B, 5A). In the mouse cultures, it is clear that the increase of MAP2 
positive cells cannot be caused by an effect on astrocyte proliferation, because this number of GFAP 
positive cells was low and did not vary after treatment with N-CAM. When either CNTF or FCS was added 2 d after

Figure 4. Stimulation of differentiation of rat and mouse hippocampal neural progenitor cells toward a neuronal phenotype by N-CAM. A, Quantitation of differentiation of rat hippocampal progenitor cells. Cells were grown for 4 d on poly-l-lysine- and laminin-coated glass multichamber slides in NB/B27 media in the presence of bFGF alone (20 ng/ml) and then treated for 3 d with N-CAM (10 μg/ml) or left untreated. In each experiment, the number of MAP2 
positive cells and GFAP 
positive cells is shown as a percentage of the total number of cells. A minimum of 200 cells in five different fields per condition were counted. The values are expressed as the average ± SD from a representative experiment. B, Immunocytochemistry for MAP2 (Texas Red) and GFAP (FITC) showing N-CAM induction of MAP2 
positive cells and CNTF or FCS induction of GFAP 
positive cells in mouse progenitor cell culture after treatment for 3 d with N-CAM (10 μg/ml), CNTF (100 ng/ml), or FCS (10%). Cells were counterstained with DAPI.

Figure 5. Comparison of the effect of N-CAM, BDNF, NT-3, IGF-I, PDGF, and CNTF on the differentiation of hippocampal progenitor cells. A, The protocol was exactly the same as that used in Figure 4. BDNF, NT-3, IGF-I, PDGF, and CNTF were used at 100 ng/ml, N-CAM was used at 10 μg/ml, and factors were added in the presence of bFGF (20 ng/ml) after 4 d of culture of mouse hippocampal progenitor cells (**p < 0.001; *p < 0.05; Student’s t test). B, Cells were cultured for 4 d in the presence of bFGF, after which BDNF (100 ng/ml) or N-CAM (10 μg/ml) was added without bFGF and the cultures were grown for an additional 3 d. The counting was also done as described in Figure 4, and the values represent the average ± SD from a representative experiment (**p < 0.01; *p < 0.05; Student’s t test).

N-CAM, the number of astrocytes increased (78-fold by CNTF, from 0.33 to 26%, and 69-fold by FCS, from 0.33 to 23%), but the number of MAP2 
positive cells was the same as when N-CAM was added alone (data not shown). These findings suggest that MAP2 
positive cells induced by N-CAM did not dedifferentiate and that N-CAM and CNTF or FCS may act on separate populations. Together the experiments on mouse and rat cultures indicate that N-CAM increases the differentiation of progenitor cells to a neuronal lineage in addition to its ability to reduce astrocyte proliferation.

Comparison of the effects of N-CAM and growth factors on the differentiation of mouse and rat hippocampal neural progenitor cells

The effect of N-CAM on the differentiation of hippocampal progenitor cells was compared with that of growth factors reported to stimulate the differentiation of neural progenitor cells toward specific lineages. In the presence of bFGF (Fig. 5A), the separate addition of either BDNF, NT-3, PDGF, or IGF-I did not
significantly enhance neuronal differentiation of the hippocampal progenitor cells over that seen in untreated cultures, but N-CAM treatment resulted in significant differentiation. In contrast, in the absence of bFGF (Fig. 5B), the addition of BDNF alone increased the number of MAP2⁺ cells, consistent with previous reports (Ahmed et al., 1995; Vicario-Abejon et al., 1995). Therefore, in the presence of the mitogen bFGF, N-CAM is at least as potent an inducer of neuronal differentiation as the growth factors reported previously to affect stem cell differentiation in the absence of bFGF.

**N-CAM affects the proliferation and differentiation of progenitor cells lacking N-CAM**

We investigated whether N-CAM homophilic binding was required for N-CAM to affect the proliferation and differentiation of neural progenitor cells using cell cultures derived from mice lacking N-CAM (Holst et al., 1998). Cells were prepared from E16 to E17 heterozygous (+/−) or N-CAM knock-out (−/−) mice and incubated with 10 µg/ml N-CAM, Ig I–II, or Ig III, after 2 d in culture as described in Materials and Methods. BrdU incorporation was measured over the last 24 hr of treatment. Each value represents the average ± SEM of a minimum of three experiments (***p < 0.001; Student’s t test).

**Figure 6.** Inhibition of proliferation of hippocampal progenitor cells from N-CAM heterozygous (+/−) and knock-out (−/−) mice. Neural progenitor cells were prepared from the hippocampi of N-CAM heterozygous (+/−) or N-CAM knock-out (−/−) mice and incubated with 10 µg/ml N-CAM, Ig I–II, or Ig III, after 2 d in culture as described in Materials and Methods. BrdU incorporation was measured over the last 24 hr of treatment. Each value represents the average ± SEM of a minimum of three experiments (***p < 0.001; Student’s t test).

**Figure 7.** Binding of N-CAM and extracellular N-CAM to live hippocampal progenitor cells from N-CAM knock-out mice. Cells were treated with N-CAM, the extracellular domain of N-CAM, and the recombinant Ig III domain (10 µg/ml) for 2 hr at 37°C. The cells were washed with PBS, fixed with 4% PFA, and processed for immunocytochemistry using antibodies for N-CAM and N-CAM domains and FITC-labeled secondary antibodies as described in Materials and Methods. Nuclei were revealed by counterstaining with DAPI.

**Mechanism of action of N-CAM: evidence of a heterophilic ligand**

To establish an assay for evaluating heterophilic binding, N-CAM and recombinant domains of N-CAM were allowed to bind to live cells lacking N-CAM. The bound molecules were revealed after fixation and detection with specific antibodies (Ranheim et al., 1996) (Fig. 7). N-CAM and the extracellular domain of N-CAM bound to the KO cells in vitro, whereas Ig III showed no binding (Fig. 7). Preabsorption of the N-CAM solution with N-CAM antibodies linked to beads removed the binding molecules (Fig. 7). The proteins were revealed without permeabilization of the cells, indicating that they were bound to the cell surface. Addition of 0.02% sodium azide, which blocks internalization processes, did not alter the pattern of N-CAM binding, further suggesting that N-CAM-positive staining is located at the cell surface.

Because heparin, heparan sulfate proteoglycans (HSPG), and chondroitin sulfate proteoglycans (CSPG) have been shown to interact with N-CAM (Cole et al., 1986; Friedlander et al., 1994), heparin and chondroitin sulfate were tested as competitors for N-CAM binding to the surface of live KO cells. Heparinase was also used to prevent possible interactions of exogenously added N-CAM with HSPG present on the cell surface. Cells were preincubated with heparin, heparinase, or chondroitin sulfate before N-CAM treatment. None of the treatments prevented...
N-CAM binding (Fig. 8). Oligosaccharides were used to test whether carbohydrate binding was involved as has been suggested for N-CAM and L1 interactions (Horstkorte et al., 1993). Pretreatment of N-CAM KO cells with N- and O-glycosidases had no effect on subsequent N-CAM binding (Fig. 8).

To confirm that N-CAM was not interacting with heparin, HSPG, or CSPG to affect proliferation, the same reagents used in the binding experiments were used in the proliferation assays (Fig. 9). None of these molecules was able to reduce the inhibitory activity of N-CAM on progenitor cell proliferation (Fig. 9). Consistent with the inability of heparin or heparan sulfate to affect proliferation, Ig I–II, which contains the Ig II domain responsible for the interaction of N-CAM with HSPG (Cole and Akeson, 1989), was inactive (Fig. 6). These findings suggest that heparan sulfate or chondroitin sulfate proteoglycans or cell surface oligosaccharides were not involved in the binding of N-CAM to the cell surface, further supporting the presence of a novel N-CAM heterophile.

We also excluded the possibility that N-CAM could bind either of the mitogens bFGF or EGF and thereby influence cell proliferation. Preincubation of the culture medium with beads coated with N-CAM followed by treatment of the cells with the eluate showed no reduction in basal proliferation or in the ability of N-CAM to inhibit proliferation, suggesting that N-CAM was not interacting with and therefore depleting bFGF or EGF from the medium (data not shown). N-CAM has been reported to interact with the FGF receptor (Williams et al., 1994; Doherty and Walsh, 1996). An antibody against the FGF receptor that was shown in these studies to block the N-CAM effect on neurite outgrowth (Williams et al., 1994; Doherty and Walsh, 1996) was used to test the possible interaction of the FGF receptor with N-CAM. The application of the FGF receptor antibody alone reduced cell proliferation in a dose-dependent manner. When used at a concentration that did not dramatically affect proliferation but showed FGF receptor immunostaining (data not shown), the inhibition of proliferation by N-CAM was not prevented by the addition of this FGF receptor antibody (Fig. 9).

**DISCUSSION**

We have investigated the effect of N-CAM on neural progenitor cell proliferation and differentiation. In previous studies, we found that N-CAM affected the proliferation of astrocytes and NF-κB signaling in both astrocytes and neurons. We therefore hypothesized that N-CAM might have a significant effect on neural progenitor cells. The studies reported here support this idea but indicate that the mechanism of N-CAM action is significantly different. In astrocytes and neurons, N-CAM apparently can signal via N-CAM itself by a mechanism that is influenced by the first three Ig domains. Its effect on neuronal precursors, however, appears to involve signaling via an unidentified heterophilic ligand. In the presence of either bFGF or EGF, both of which favor proliferation of neural progenitor cells, addition of N-CAM decreased cell proliferation. The inhibition of proliferation by N-CAM was not restricted to hippocampal progenitor cells but also occurred in neonatal cerebellar rat progenitor cell cultures and normal human neural progenitor cells (M.-C. Amoureux and K. L. Crossin, unpublished observations). N-CAM binding to the surface of several neural cell types can therefore inhibit their proliferation.

In addition to inhibiting cell proliferation, N-CAM efficiently promoted neuronal differentiation in the presence of bFGF. Previous studies have shown that a variety of neurotrophins and growth factors, including BDNF, NT-3, CNTF, leukemia inhibitory factor, IGF-I, and PDGF, enhance neuronal stem cell differ-
entiation after bFGF removal (Ahmed et al., 1995; Ghosh and Greenberg, 1995; Temple and Qian, 1995; Vicario-Abejon et al., 1995; Kahn et al., 1997; Williams et al., 1997; Arsenijevic and Weiss, 1998; Koblar et al., 1998; Zigova et al., 1998). However, the present results demonstrate that these factors had little effect in the presence of bFGF, in contrast to the robust effect of N-CAM. One of the reported effects of neurotrophins is to decrease the amount of apoptosis of cortical progenitors and to promote neuronal survival in the absence of growth factors (Kirschbaum and Goldman, 1995; Wade et al., 1999). Because little apoptosis was observed in the presence of bFGF, the possible effects of N-CAM on cell survival were not measurable in our experimental paradigm. However, the previously reported ability of BDNF to enhance neural differentiation in the absence of bFGF was also observed in this study and might reflect the survival-promoting activity of BDNF in the absence of bFGF or the ability of BDNF to enhance differentiation (Ahmed et al., 1995). Moreover, it has been suggested that different mitogens promote the proliferation or the survival of particular progenitor populations (Liillien, 1998). In the case of N-CAM, a specific mitogen did not seem to be critical, because N-CAM inhibited both bFGF- and EGF-stimulated proliferation. Moreover, N-CAM was equally effective in promoting neuronal differentiation in the presence or absence of bFGF. These results indicate that N-CAM did not achieve its effects on proliferation and differentiation by acting on a specific subpopulation of progenitors or by influencing particular growth factor receptor signaling as reported in other in vitro systems (Williams et al., 1994; Doherty and Walsh, 1996).

The increased number of MAP2+ cells stimulated by the presence of N-CAM could result from several mechanisms. It is likely that N-CAM decreased the number of GFAP+ cells in rat cell culture at least partially because of its inhibitory effect on astrocyte proliferation as shown in previous studies (Sporns et al., 1995; Krushel et al., 1998), although it is possible that N-CAM treatment could have induced these cells to become MAP2+ neuroblasts as reported previously for subventricular zone GFAP+ stem cells (Doetsch et al., 1999). In any case, the increase in the number of MAP2+ cells induced by N-CAM in rat neural progenitors was much greater than could be accounted for simply by the decrease in GFAP+ cells. The use of mouse progenitor cells allowed us to verify this conclusion unambiguously. Cultures of these cells displayed almost no GFAP+ cells (0.33%) compared with that in rat hippocampal cells (12%) in the same culture conditions. The increase of MAP2+ cells after N-CAM treatment of mouse cells resulted therefore from the differentiation of MAP2-GFAP- cells that were present. It is also unlikely that the increase in the ratio of MAP2+ cells could have been caused by a stimulation of the proliferation of the MAP2+ neuroblast population in view of the dramatic global inhibitory effect of N-CAM on proliferation. In addition, N-CAM did not stimulate proliferation in the absence of bFGF. It can therefore be concluded that N-CAM acts on a stage in the differentiation process rather than by producing a secondary effect on MAP2+ or GFAP+ cells.

The relationship between the inhibition of proliferation and the increase in differentiation observed here requires further study. There is no clear evidence that the two events, cell cycle arrest and differentiation, are interdependent in neural stem and progenitor cells, and indeed, evidence to the contrary exists. For example, the degree of primitivity of a stem cell seems not to be correlated with its mitotic properties. Some neural stem cells are mitotically quiescent, whereas others divide rapidly; intermediary progenitors proliferate faster than do pluripotent stem cells (Morrison et al., 1997). Interestingly, the same situation is true for hematopoietic stem cells, in which the most primitive stem cells are highly quiescent but can be maintained in long-term culture (Hao et al., 1996). In addition, even though a fraction of the neural stem cells of the subventricular zone undergoes apoptosis in vivo (Morshead and van der Kooy, 1992), many neural stem cells remain quiescent and yet do not differentiate (Morshead et al., 1994). It is probable that various cell cycle-controlling factors are involved in a multistep manner to lead a cell to exit the cell cycle and enter a differentiation program (Robertson and Levitt, 1999; Scheffler et al., 1999). These observations further support the idea that N-CAM may instructively induce progenitor differentiation, independent of its effect on proliferation.

The present study raises the possibility that N-CAM is an endogenous regulator of progenitor cell proliferation and differentiation. Membrane-associated factors have been suggested previously to play a role in the control of progenitor cell proliferation. For example, stem cells grown as neurospheres seem to proliferate faster than the same cells plated on a substrate (Reynolds and Weiss, 1996). Other studies indicate that contact with some cell types favors proliferation whereas interactions with other cell types are inhibitory to proliferation (Temple and Davis, 1994). Removal of PSA from oligodendrocyte preprogenitors, which increases cell–cell interactions, has also been reported recently to increase the differentiation of these cells (Nait-Oumesmar et al., 1999). Delta and Notch are also cell membrane proteins (ligand and receptor, respectively) that control progenitor differentiation. Their interaction prevents Notch-expressing cells from differentiating by inhibiting the production of neurogenic transcriptional regulators (Ohtsuka et al., 1999). Whether an increase of neurogenic gene expression in progenitor cells occurs after N-CAM binding remains to be investigated.

These results indicate that molecular signaling events underlying the cell–cell interaction mediated by N-CAM may participate in progenitor cell proliferation and differentiation. The mechanism by which N-CAM modulates neural differentiation remains to be determined. N-CAM may generate intracellular signals directly by interacting with a heterophilic receptor or may perturb preexisting endogenous molecular interactions and thereby prevent rather than mimic the normal signaling events generated by N-CAM interactions. It is possible that N-CAM activates the same intracellular pathways in progenitor cells as do the neurotrophins, which bind to their tyrosine kinase receptors, the Trks (Lachyankar et al., 1997). Recent studies suggest that N-CAM signaling involves nonreceptor tyrosine kinase activation (Beggs et al., 1997; Choi et al., 1999) and that this activation may influence multiple intracellular signaling cascades. Because N-CAM counteracts the mitogenic effects of bFGF (Ray and Gage, 1994; Gritti et al., 1996) (present results), it is also likely that N-CAM signal pathways alter signaling via the FGF receptor, N-CAM interactions. It is possible that N-CAM activates the same intracellular pathways in progenitor cells as do the neurotrophins, which bind to their tyrosine kinase receptors, the Trks (Lachyankar et al., 1997). Recent studies suggest that N-CAM signaling involves nonreceptor tyrosine kinase activation (Beggs et al., 1997; Choi et al., 1999) and that this activation may influence multiple intracellular signaling cascades. Because N-CAM counteracts the mitogenic effects of bFGF (Ray and Gage, 1994; Gritti et al., 1996) (present results), it is also likely that N-CAM signal pathways alter signaling via the FGF receptor, which has been reported to interact with N-CAM in other cellular systems (Doherty and Walsh, 1996). Such a mechanism of antagonism of growth factor receptor signals has been suggested in previous studies (Ghosh and Greenberg, 1995). Distinct signaling pathways from N-CAM, Trks, and Notch may each influence the balance between stimulatory and inhibitory signals leading to differentiation.

Identification of the ligand to which N-CAM binds to exert its effects on progenitor cells will help to define the mechanism of N-CAM action. Binding of N-CAM to live cells from N-CAM
KO mice provides an assay to evaluate cellular extracts for such heterophiles. Moreover, the tissue from knock-out animals provides an excellent source of protein to identify heterophiles without interference from N-CAM homophilic binding. Possible heterophilic ligands, such as membrane proteins, particularly homologs of N-CAM such as O-CAM (Schwob and Gottlieb, 1988; Yoshihara et al., 1997), basement membrane proteins, ECM proteins, the protein core of proteoglycans, or new peptide ligands (Ronn et al., 1999), may bind exogenous N-CAM. However, we present evidence that the previously proposed heterophilic ligands for N-CAM, including the heparan or chondroitin sulfate part of proteoglycans or FGF receptors at the cell surface (Cole et al., 1986; Friedlander et al., 1994; Williams et al., 1994; Doherty and Walsh, 1996), are not the ligands by which N-CAM alters progenitor cell proliferation or differentiation.

Whether N-CAM binding affects the genesis or differentiation of neural progenitors in vivo is of critical interest, and resolution of this issue may suggest mechanisms of controlling stem cell states during development and in adult animals after transplantation. PSA-N-CAM is present endogenously in neurogenic regions, such as the hippocampus (Seki and Arai, 1991) and subventricular zone (Key and Akeson, 1991; Thomas et al., 1996). Limited cell–cell interactions because of the presence of PSA-N-CAM could, in concert with growth factors, control neural progenitor cell proliferation and differentiation. The specific spatio-temporal distribution of N-CAM and N-CAM ligands may turn out to represent significant factors in the genesis of cells of the nervous system.

REFERENCES


Krishal LA, Sporns O, Cunningham BA, Crossin KL, Edelman GM...


