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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

Neural stem cells have been the object of increasing attention for their potential use in cell replacement or gene therapy (Pincus et al., 1998). Extensive neurogenesis occurs throughout development, but only recently has it been demonstrated that the adult brain of vertebrates also contains a source of proliferative stem cells (Richards et al., 1992; Barnea and Nottebohm, 1996; Kempermann et al., 1997, 1998a,b; Palmer et al., 1997). Attempts to define a neural stem cell have been made, but the lack of appropriately specific markers and the pace of new discoveries (Doetsch et al., 1999; Johansson et al., 1999) leave only two unambiguous criteria: self-renewal and multipotentiality. Cells from different regions of the CNS of primates and rodents have been isolated and shown to proliferate in the presence of growth factors (Reynolds and Weiss, 1992, 1996; Reynolds et al., 1992; Ray and Gage, 1994; Williams and Price, 1995; Gritti et al., 1996). The cells can generate cells of glial and neuronal lineages *in vitro* in the presence of particular neurotrophic factors (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) or *in vivo* after transplantation (Chuah et al., 1991; Campbell et al., 1995; Craig et al., 1996; Carpenter et al., 1997; Winkler et al., 1998). The role of cell surface molecules other than receptors for neurotrophic factors and growth factors remains an important subject for study.

The neural cell adhesion molecule (N-CAM) is one of many CAMs and extracellular matrix (ECM) proteins that mediate cell interactions and modulate developmental processes including neuronal migration, neurite extension, and gene expression. CAMs also participate in neural regeneration, neurite fasciculation, and synaptogenesis in the mature nervous system (for review, see Edelman and Crossin, 1991; Lynch et al., 1991; Doherty and Walsh, 1996). N-CAM may be important in neural stem cell biology on the basis of recent studies of the rostral migratory pathway, in which neural stem cells migrate to populate the olfactory bulb. Polysialic acid (PSA), a large, negatively charged sugar moiety carried by N-CAM that reduces its adhesive efficacy (Seki and Arai, 1991), N-CAM, and laminin are abundantly expressed along this pathway from the subventricular zone to the olfactory bulb (Key and Akeson, 1991; Lois and Alvarez-Buylla, 1994; Lois et al., 1996; Thomas et al., 1996), suggesting that the place-dependent expression of these molecules may contribute to neural stem cell migration and differentiation. Indeed, the PSA-rich rostral migratory pathway has been proposed to promote neural stem cell migration to the olfactory bulb (Rousselot et al., 1995). Various N-CAM knock-out mouse models (Tomasiewicz et al., 1993; Cremer et al., 1994; Holst et al., 1998) and a PSA-depleted mouse model (Ono et al., 1994) present a similar phenotype that includes a reduced olfactory bulb, which has been attributed to decreased migration of the subventricular zone cells to their target. PSA-N-CAM is also found in other areas where neural proliferation occurs, such as the hippocampus (Seki and Arai, 1991), raising the possibility that the decreased membrane contacts in the presence of PSA-N-CAM (Rutishauser et al., 1988; Acheson et al., 1991) are favorable to neurogenesis.

The involvement of neural CAMs in the control of neural stem

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cell proliferation and differentiation has not been studied directly. We reported previously that N-CAM binding inhibits astrocyte proliferation *in vitro* and *in vivo* after a lesion (Krushel et al., 1995, 1998; Sporns et al., 1995) and decreases the proliferation of other N-CAM-expressing cell lines (Krushel et al., 1998). We hypothesized that N-CAM, acting in conjunction with growth factors, may play a role in influencing the balance between proliferation and differentiation of neural stem cells. The present study investigated the effect of N-CAM on hippocampal progenitor cell proliferation and differentiation. The findings suggest that N-CAM and its ligands play a role in controlling the proliferation of neural progenitor cells and directing their differentiation toward a neuronal lineage.

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 $500 \times g$, and the buffer was removed. To dissociate the tissue into single cells, we incubated it for 20 min at 37°C in 0.15% 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 $500 \times g$. 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 immunoaffinity 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; 100 μl per well was seeded in 96-well plates (Packard, Meridian, CT) coated with poly-L-lysine and laminin (Sigma). After 2 d in NB/B27 supplemented with 20 ng/ml bFGF, the cells were treated with various N-CAM protein reagents for 48 hr. During the last 24 hr of this period, 5-bromo-2′-deoxyuridine (BrdU; 10 μM) was added. At the end of the 48 hr, cells were fixed in 4% paraformaldehyde (PFA) for 30 min. BrdU was quantified using the chemiluminescence 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), cells were fixed with 4% PFA (Sigma). The TUNEL assay was used to assess apoptosis. Briefly, fragmented DNA was extended by the terminal deoxynucleotide transferase (250 U/ml; Boehringer Mannheim) that incorporated chromatide bodipy-FL-14-dUTP (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 0.05N 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 staining, 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. Nestin and BrdU 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-I), 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 527) 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. 1*A*). At this time, ~70% of the cells incorporated BrdU over 24 hr (Fig. 1*B*). bFGF or a combination of both epidermal growth factor (EGF) and bFGF supported cell proliferation, whereas

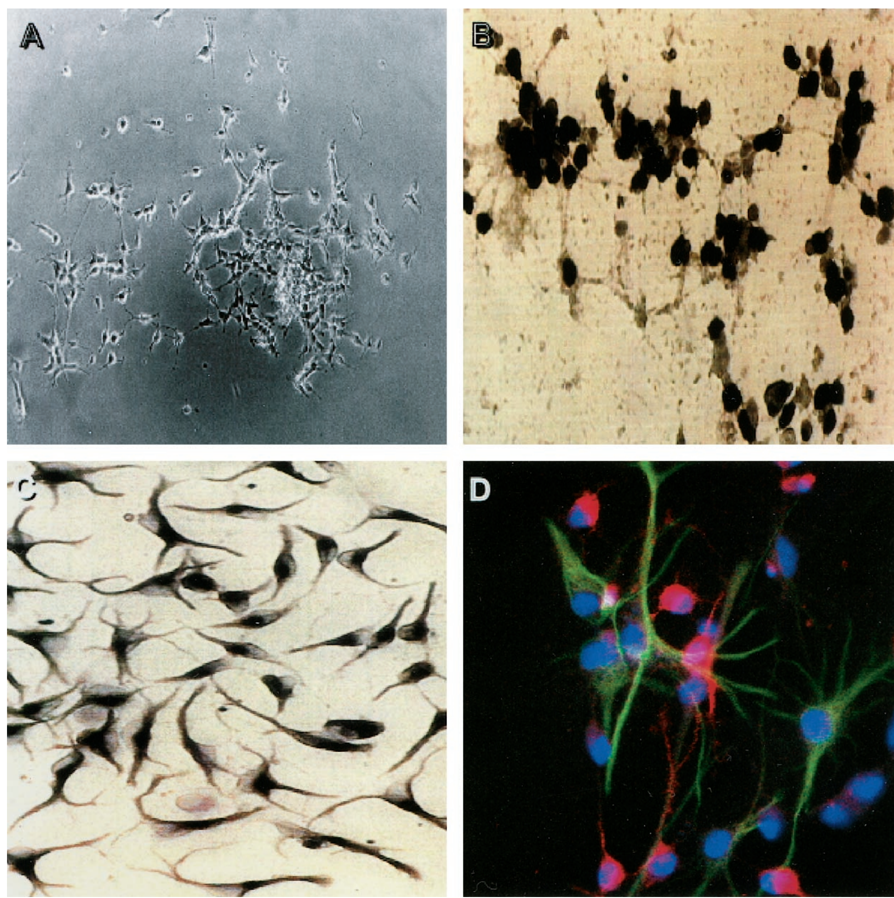

Figure 1. Characterization of primary E17–E18 rat hippocampal progenitor cells. *A*, Bright-field view of proliferating rat hippocampal cells cultured on poly-L-lysine- and laminin-coated substrates in NB/B27 medium in the presence of 20 ng/ml bFGF after 4 d in culture. *B, C*, BrdU incorporation (*B*) and immunolabeling (*C*) of the intermediate filament protein nestin. *D*, Phenotype of cells cultured for 4 d in NB/B27 + 20 ng/ml bFGF followed by 2 d of culture in the same medium without bFGF to stimulate differentiation. Cells were double-labeled for GFAP (FITC; *green*) and MAP2 (Texas Red; *red*) and counterstained with the nuclear stain DAPI (*blue*). Magnification: *A*, 10×; *B—D*, 40×.

EGF alone was an effective but less potent mitogen (data not shown). The cells were unipolar or bipolar with short extensions (Fig. 1*A*), and 95% of the cells expressed nestin, an intermediate filament protein present in neural stem cells and progenitors (Lendhal et al., 1990) (Fig. 1*C*). 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. 1*D*; 25% $GFAP^+$ and 70% $MAP2^+$). S100 antibodies stained all $GFAP^+$ cells and no others. Approximately 1% of the cells expressed the oligodendrocyte marker 04 (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. Amoreaux 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. 2*A*). A concentration of N-CAM > 1.25 μg/ml was necessary to obtain measurable inhibition, and the IC_{50} was determined to be $\sim$1.5 μg/ml. Preabsorption of the N-CAM solution with beads coated with monoclonal antibody against N-CAM abolished the inhibitory activity (Fig. 2*B*), 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 polylysine and/or laminin substrate (data not shown). N-CAM (10 μg/ml) could also inhibit EGF-stimulated progenitor proliferation by 72 $\pm$ 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. 2*B*). The native extracellular domain inhibited cell proliferation, whereas the recombinant Ig III domain did not. It was surprising that neither Ig III (Fig. 2*B*) 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-

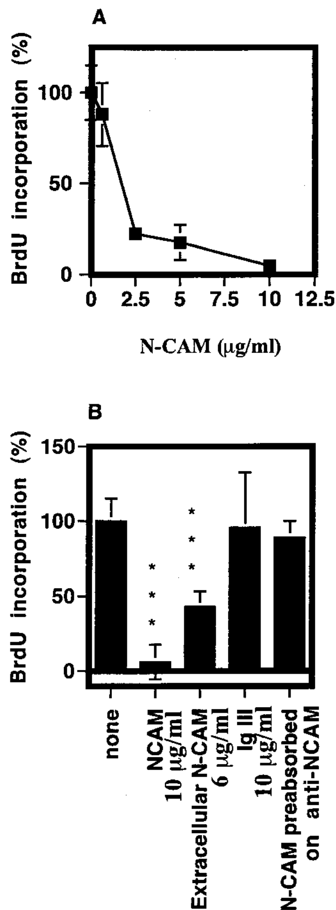

Figure 2. Inhibition of rat hippocampal progenitor cell proliferation by N-CAM. *A*, Dose response of the effect of N-CAM on rat hippocampal progenitor cell proliferation measured by BrdU incorporation stimulated by 20 ng/ml bFGF. The *values* indicated on the *graph* represent the relative BrdU incorporation compared with that of untreated cultures. *Values* represent the average ± SD of a minimum of two experiments. *B*, BrdU incorporation stimulated by 20 ng/ml bFGF measured after treatment with N-CAM, the extracellular domain of N-CAM, the recombinant Ig III domain of N-CAM, or N-CAM preabsorbed on an N-CAM monoclonal antibody as described in Materials and Methods. The *values* are normalized to the BrdU incorporation in untreated cultures. Each *value* represents the average ± SEM of a minimum of three experiments (***$p < 0.001$; Student's *t* test).

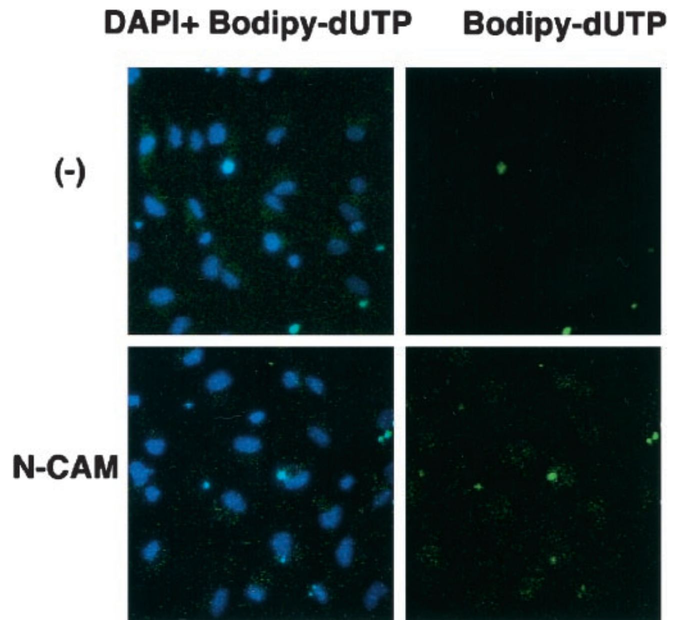

Figure 3. Absence of apoptotic cell death after N-CAM treatment. Hippocampal cells were plated in NB/B27 with 20 ng/ml bFGF for 48 hr and treated for 2 additional days with 10 μg/ml N-CAM. Apoptosis was assessed using the TUNEL method as described in Materials and Methods. *Green* fluorescent nuclei correspond to apoptotic cells in which the terminal transferase has incorporated fluorescent dUTP (*Bodipy-dUTP*) into fragmented DNA. All the cells were counterstained with the nuclear stain DAPI shown in *blue*. A minimum of 10 independent fields was used to assess the percentage of apoptotic cells (see Results).

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. 1*D*). 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. 4*A*). 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. 4*B*, 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. 4*B*, 5). However, in agreement with the findings using rat cultures, N-CAM treatment significantly increased the percentage of MAP2$^+$ cells (Figs. 4*B*, 5). Although N-CAM did

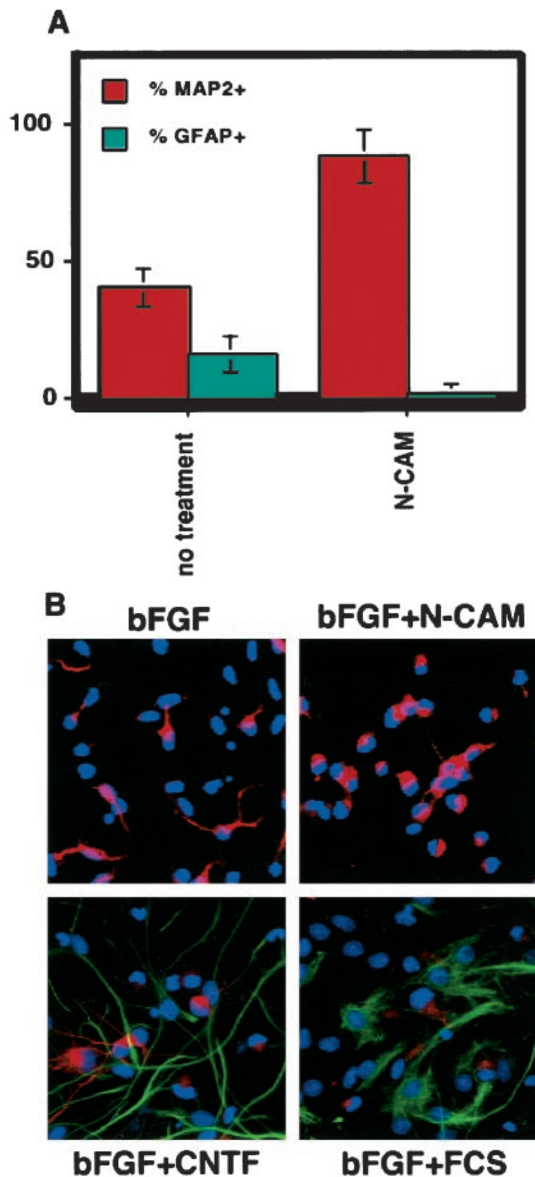

Figure 4. Stimulation of differentiation of rat and mouse hippocampal neural progenitor cells toward a neuronal phenotype by N-CAM. *A*, Quantitation of differentiation of rat hippocampal progenitor cells. Cells were grown for 4 d on poly-L-lysine- and laminin-coated glass multichamber slides in NB/B27 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$^+$ cells and GFAP$^+$ 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$^+$ cells and CNTF or FCS induction of GFAP$^+$ 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.

not affect the percentage of astrocytes from mouse progenitor cell cultures (Fig. 5*A*), a GFAP$^+$ population could be induced by the addition of CNTF or FCS (Figs. 4*B*, 5*A*). In the mouse cultures, it is clear that the increase of MAP2$^+$ cells cannot be caused by an effect on astrocyte proliferation, because this number of GFAP$^+$ cells was low and did not vary after treatment with N-CAM. When either CNTF or FCS was added 2 d after

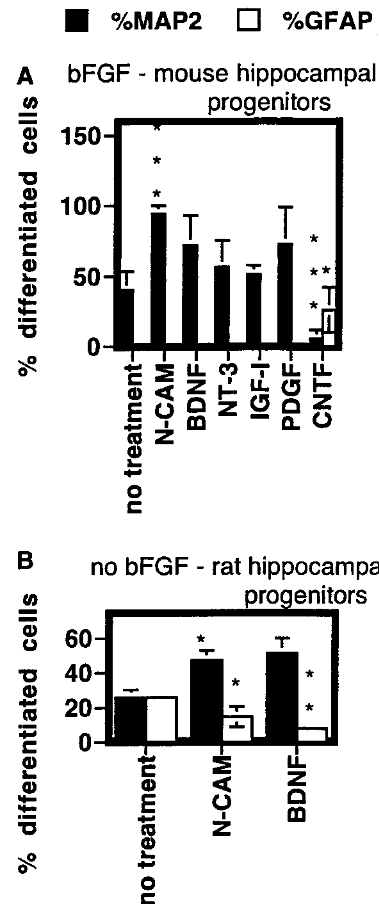

Figure 5. Comparison of the effect of N-CAM, BDNF, NT-3, IGF-I, PDGF, and CNTF on the differentiation of hippocampal progenitor cells. *A*, The protocol was exactly the same as that used in Figure 4. BDNF, NT-3, IGF-I, PDGF, and CNTF were used at 100 ng/ml, N-CAM was used at 10 μg/ml, and factors were 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$^+$ cells was the same as when N-CAM was added alone (data not shown). These findings suggest that MAP2$^+$ 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. 5*A*), the separate addition of either BDNF, NT-3, PDGF, or IGF-I did not

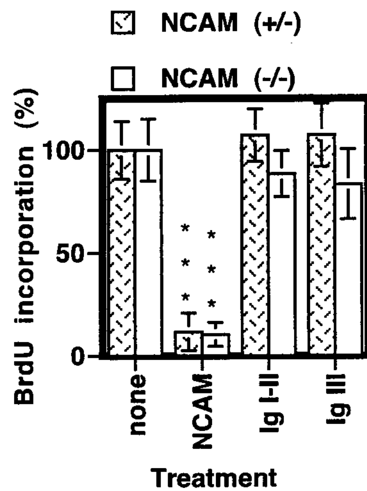

Figure 6. Inhibition of proliferation of hippocampal progenitor cells from N-CAM heterozygous (+/−) and knock-out (−/−) mice. Neural progenitor cells were prepared from the hippocampi of N-CAM heterozygous (+/−) or N-CAM knock-out (−/−) mice and incubated with 10 μg/ml N-CAM, Ig I–II, or Ig III, after 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).

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. 5*B*), 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 (+/−) and homozygous (−/−) N-CAM KO mice. Cells from N-CAM +/− animals expressed N-CAM at their surface, whereas those from N-CAM −/− animals did not (data not shown), and cells from either origin incorporated BrdU after multiple passages, indicating that they could be subcultured and remain proliferative. They were further characterized as described below.

N-CAM and recombinant N-CAM fragments were added to the culture medium containing bFGF (20 ng/ml), and proliferation was measured by BrdU incorporation. N-CAM strongly reduced the proliferation of progenitor cells derived from N-CAM +/− mice or N-CAM −/− mice (Fig. 6). N-CAM recombinant fragments (Fig. 6) or a polyclonal antibody against N-CAM (data not shown) had little effect, in contrast to studies on astrocytes in which N-CAM fragments and antibodies all reduced cell proliferation (Sporns et al., 1995; Krushel et al., 1998). N-CAM also effectively induced the differentiation of N-CAM KO hippocampal progenitor cells to the same extent as in N-CAM wild-type or rat cell cultures. The percentage of

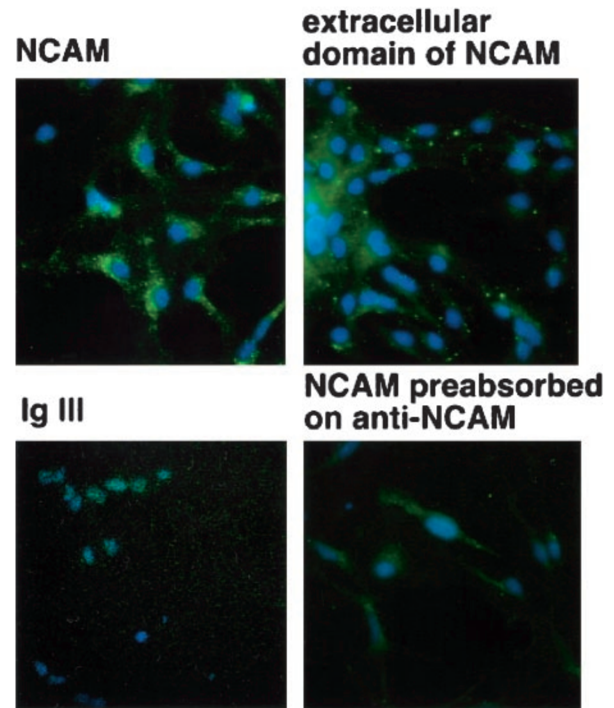

Figure 7. Binding of N-CAM and extracellular N-CAM to live hippocampal progenitor cells from N-CAM knock-out mice. Cells were treated with N-CAM, the extracellular domain of N-CAM, and the recombinant Ig III domain (10 μg/ml) for 2 hr at 37°C. The cells were washed with PBS, fixed with 4% PFA, and processed 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.

MAP2$^+$ cells was increased from 18 ± 7.0 to 51 ± 7.5%. These data strongly support the involvement of a heterophilic ligand in the effects of N-CAM on neural progenitor cell proliferation and differentiation.

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

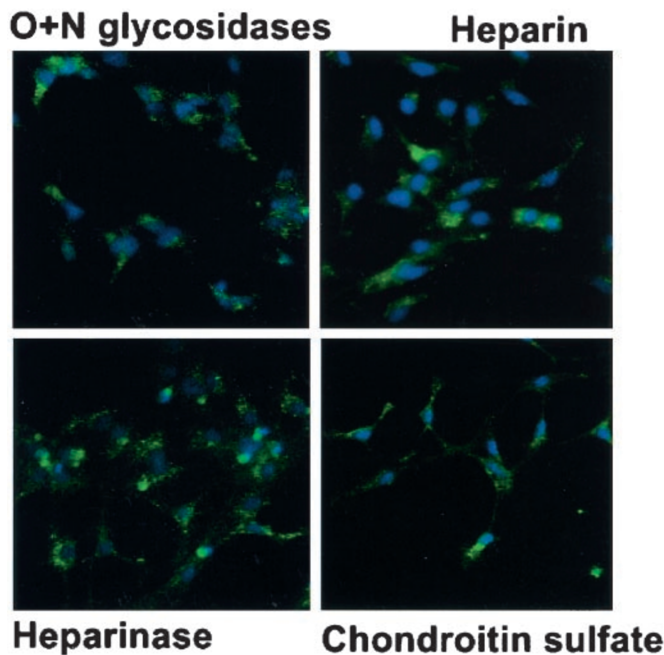

Figure 8. Saccharides and saccharide-modifying enzymes do not affect N-CAM binding to knock-out progenitor cells. Cells were pretreated (1 hr) with heparin (0.01 mg/ml), chondroitin sulfate (0.01 mg/ml), heparinase (0.01 U/ml), or glycosidases (4 U/ml; N-glycosidase endo F and O-glycosidase) and then treated with N-CAM (10 μg/ml) for 2 hr at 37°C. The cells were washed with PBS, fixed with 4% PFA, and processed for immunocytochemistry for N-CAM, as described in Materials and Methods.

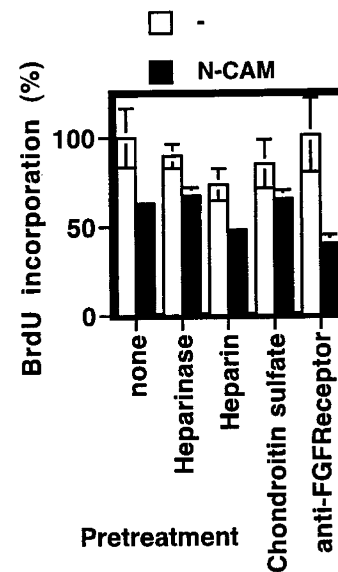

Figure 9. N-CAM inhibitory activity on proliferation is not affected by known N-CAM heterophiles. The protocol was the same as that used in Figure 2, with N-CAM applied at 2 μg/ml. Cells were pretreated (1 hr) with heparin (0.01 mg/ml), chondroitin sulfate (0.01 mg/ml), heparinase (0.01 U/ml), anti-FGF receptor (1:200), or glycosidases (4 U/ml; N-glycosidase endo F and O-glycosidase). The *values* represent the BrdU incorporation in a representative experiment. *Values* are expressed as the average ± SD of a minimum of three measurements.

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 as yet 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. Amoreaux 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 neural 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 (Kirschenbaum 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 (Lillien, 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, 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 spatiotemporal 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

Acheson A, Sunshine JL, Rutishauser U (1991) N-CAM polysialic acid can regulate both cell-cell and cell-substrate interactions. J Cell Biol 114:143–153.

Ahmed S, Reynolds BA, Weiss S (1995) BDNF enhances the differentiation but not the survival of CNS stem cell-derived neuronal precursors. J Neurosci 15:5765–5778.

Arsenijevic Y, Weiss S (1998) Insulin-like growth factor-I is a differentiation factor for postmitotic CNS stem cell-derived neuronal precursors: distinct actions from those of brain-derived neurotrophic factor. J Neurosci 18:2118–2128.

Azizeh BY, Cribbs DH, Kreng VM, Cotman CW (1998) Cross-linking of N-CAM receptors on neurons induces programmed cell death. Brain Res 796:20–26.

Barnea A, Nottebohm F (1996) Recruitment and replacement of hippocampal neurons in young and adult chickadees: an addition to the theory of hippocampal learning. Proc Natl Acad Sci USA 93:714–718.

Beggs HE, Baragona SC, Hemperly JJ, Maness PF (1997) NCAM140 interacts with the focal adhesion kinase p125 fak and the src-related tyrosine kinase p59 (fyn). J Biol Chem 272:8310–8319.

Campbell K, Olsson M, Bjorklund A (1995) Regional incorporation and site-specific differentiation of striatal precursors transplanted to the embryonic forebrain ventricle. Neuron 15:1259–1273.

Carpenter MK, Winkler C, Fricker R, Emerich DF, Wong SC, Greco C, Chen E-Y, Chu Y, Kordower JH, Messing A, Bjorklund A, Hammang JP (1997) Generation and transplantation of EGF-responsive neural stem cells derived from GFAP-hNGF transgenic mice. Exp Neurol 148:187–204.

Choi J, Krushel LA, Edelman GM, Crossin KL (1999) Neural cell adhesion molecule (N-CAM) activates NF-kappaB through SRC-family tyrosine kinases and PKC in astrocytes and neurons. Soc Neurosci Abstr 25:1697.

Chuah MI, David S, Blaschuk O (1991) Differentiation and survival of rat olfactory epithelial neurons in dissociated cultures. Dev Brain Res 60:123–132.

Cole CJ, Loewy A, Glaser L (1986) Neuronal cell-cell adhesion depends on interaction of N-CAM with heparin-like molecules. Nature 320:445–447.

Cole GJ, Akeson R (1989) Identification of a heparin binding domain of the neural cell adhesion molecule N-CAM using synthetic peptides. Neuron 2:1157–1165.

Craig CG, Tropepe V, Morshead CM, Reynolds BA, Weiss S, van der Kooy D (1996) *In vivo* growth factor expansion of endogenous subependymal neural precursor cell populations in the adult mouse brain. J Neurosci 16:2649–2658.

Cremer H, Lange R, Christoph A, Plomann M, Vopper G, Roes J, Brown R, Baldwin S, Kraemer P, Scheff S, Barthels D, Rajewsky K (1994) Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. Nature 367:455–459.

Cunningham BA, Hoffman S, Rutishauser U, Hemperly JJ, Edelman GM (1983) Molecular topography of N-CAM: surface orientation and location of sialic acid-rich and binding regions. Proc Natl Acad Sci USA 80:3116–3120.

Doetsch F, Caillé I, Lim DA, García-Verdugo JM, Alvarez-Buylla A (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97:703–716.

Doherty P, Walsh FS (1996) CAM-FGF receptor interactions: a model for axonal growth. Mol Cell Neurosci 8:99–111.

Edelman GM, Crossin KL (1991) Cell adhesion molecules: implications for a molecular histology. Annu Rev Biochem 60:155–190.

Friedlander DR, Milev P, Karthikeyan L, Margolis RK, Margolis RU (1994) The neuronal chondroitin sulfate proteoglycan neurocan binds to the neural cell adhesion molecules Ng-CAM/L1/NILE and N-CAM, and inhibits neuronal adhesion and neurite outgrowth. J Cell Biol 125:669–680.

Ghosh A, Greenberg M (1995) Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. Neuron 15:89–103.

Gritti A, Parati EA, Cova L, Frolichsthal P, Galli R, Wanke E, Faravelli L, Morassutti DJ, Roisen F, Nickel DD, Vescovi AL (1996) Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. J Neurosci 16:1091–1100.

Hao QL, Thiemann FT, Petersen D, Smogorzewska EM, Crooks GM (1996) Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population. Blood 88:3306–3313.

Hoffman S, Sorkin BC, White PC, Brackenbury R, Mailhammer R, Rutishauser U, Cunningham BA, Edelman GM (1982) Chemical characterization of a neural cell adhesion molecule purified from embryonic brain membranes. J Biol Chem 257:7720–7729.

Holst BD, Vanderklish PW, Krushel LA, Zhou W, Langdon RB, McWhirter JR, Edelman GM, Crossin KL (1998) Allosteric modulation of AMPA-type glutamate receptors increases activity of the promoter for the neural cell adhesion molecule, N-CAM. Proc Natl Acad Sci USA 95:2597–2602.

Horstkorte R, Schachner M, Magyar JP, Vorherr T, Schmitz B (1993) The fourth immunoglobulin-like domain of NCAM contains a carbohydrate recognition domain for oligomannosidic glycans implicated in association with L1 and neurite outgrowth. J Cell Biol 121:1409–1421.

Johansson CB, Momma S, Clarke DL, Risling M, Lendahl U, Frisén J (1999) Identification of a neural stem cell in the adult mammalian central nervous system. Cell 96:25–34.

Kahn MA, Huang CJ, Caruso A, Barresi V, Nazarian R, Condorelli DF, de Vellis J (1997) Ciliary neurotrophic factor activates JAK/Stat signal transduction cascade and induces transcriptional expression of glial fibrillary acidic protein in glial cells. J Neurochem 68:1413–1423.

Kempermann G, Kuhn HG, Gage FH (1997) More hippocampal neurons in adult mice living in an enriched environment. Nature 386:493–495.

Kempermann G, Brandon EP, Gage FH (1998a) Environmental stimulation of 129/SvJ mice causes increased cell proliferation and neurogenesis in the adult dentate gyrus. Curr Biol 8:939–942.

Kempermann G, Kuhn HG, Gage FH (1998b) Experience induced neurogenesis in the senescent dentate gyrus. J Neurosci 18:3206–3212.

Key B, Akeson RA (1991) Delineation of olfactory pathways in the frog nervous system by unique glycoconjugates and N-CAM glycoforms. Neuron 6:381–396.

Kirschenbaum B, Goldman SA (1995) Brain-derived neurotrophic factor promotes the survival of neurons arising from the adult rat forebrain subependymal zone. Proc Natl Acad Sci USA 92:210–214.

Koblar SA, Turnley AM, Classon BJ, Reid KL, Ware CB, Cheema SS, Murphy M, Bartlett PF (1998) Neural precursor differentiation into astrocytes requires signaling through the leukemia inhibitory factor receptor. Proc Natl Acad Sci USA 95:3178–3181.

Krushel LA, Sporns O, Cunningham BA, Crossin KL, Edelman GM

(1995) The neural cell adhesion molecule (N-CAM) inhibits astrocyte proliferation after injury to different regions of the adult rat brain. Proc Natl Acad Sci USA 92:4323–4327.

Krushel LA, Tai M-H, Cunningham BA, Edelman GM, Crossin KL (1998) Neural cell adhesion molecule (N-CAM) domains and intracellular signaling pathways involved in the inhibition of astrocyte proliferation. Proc Natl Acad Sci USA 95:2586–2592.

Lachyankar MB, Condon PJ, Quesenberry PJ, Litofsky NS, Recht LD, Ross AH (1997) Embryonic precursor cells that express Trk receptors: induction of different cell fates by NGF, BDNF, NT-3, and CNTF. Exp Neurol 144:350–360.

Lendhal U, Zimmerman LB, McKay RDG (1990) CNS stem cells express a new class of intermediate filament protein. Neuron 60:585–595.

Lillien L (1998) Neural progenitors and stem cells: mechanisms of progenitor heterogeneity. Curr Opin Neurobiol 8:37–44.

Lois C, Alvarez-Buylla A (1994) Long-distance neuronal migration in the adult mammalian brain. Science 264:1145–1148.

Lois C, Garcia-Verdugo J-M, Alvarez-Buylla A (1996) Chain migration of neuronal precursors. Science 271:978–981.

Lynch G, Bahr BA, Vanderklish PW (1991) Induction and stabilization of long-term potentiation, In: Glutamate, cell death, and memory (Ascher P, Choi D, Christen Y, eds), pp 45–60. Berlin: Springer.

Mayer-Proschel M, Kalyani AJ, Mujtaba T, Rao MS (1997) Isolation of lineage-restricted neuronal precursors from multipotent neuroepithelial stem cells. Neuron 19:773–785.

Morrison SJ, Shah NM, Anderson DJ (1997) Regulatory mechanisms in stem cell biology. Cell 88:287–298.

Morshead CM, van der Kooy D (1992) Postmitotic death is the fate of constitutively proliferating cells in the subependymal layer of the adult mouse brain. J Neurosci 12:249–256.

Morshead CM, Reynolds BA, Craig CG, McBurney MW, Staines WA, Morassutti D, Weiss S, van der Kooy D (1994) Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. Neuron 13:1071–1082.

Nait-Oumesmar B, Decker L, Lachapelle F, Avellana-Adalid V, Bachelin C, Baron-Van Evercooren A (1999) Progenitor cells of the adult mouse subventricular zone proliferate, migrate and differentiate into oligodendrocytes after demyelination. Eur J Neurosci 11:4357–4366.

Ohtsuka T, Ishibashi M, Gradwohl G, Nakanishi S, Guillemot F, Kageyama R (1999) *Hes1* and *Hes5* as Notch effectors in mammalian neuronal differentiation. EMBO J 18:2196–2207.

Ono K, Tomasiewicz H, Magnuson T, Rutishauser U (1994) N-CAM mutation inhibits tangential neuronal migration and is phenocopied by enzymatic removal of polysialic acid. Neuron 13:595–609.

Palmer TD, Takahashi J, Gage FH (1997) The adult rat hippocampus contains primordial neural stem cells. Mol Cell Neurosci 8:389–404.

Pincus DW, Goodman RR, Fraser RAR, Nedergaard M, Goldman SA (1998) Neural stem cells: a strategy for gene therapy and brain repair. Neurosurgery 42:858–867.

Ranheim TS, Edelman GM, Cunningham BA (1996) Homophilic adhesion mediated by the neural cell adhesion molecule N-CAM involves multiple immunoglobulin domains. Proc Natl Acad Sci USA 93:4071–4075.

Ray J, Gage FH (1994) Spinal cord neuroblasts proliferate in response to basic fibroblast growth factor. J Neurosci 14:3548–3564.

Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707–1710.

Reynolds BA, Weiss S (1996) Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol 175:1–13.

Reynolds BA, Tetzlaff W, Weiss S (1992) A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J Neurosci 12:4565–4574.

Richards J, Kilpatrick TJ, Bartlett PF (1992) De novo generation of neuronal cells from the adult mouse brain. Proc Natl Acad Sci USA 89:8591–8595.

Robertson K, Levitt P (1999) Transient inhibition of cell cycle alters progenitor cell development in embryonic cerebral cortex explants. Soc Neurosci Abstr 25:253.

Ronn L, Olsen M, Ostergaard S, Kiselyov V, Berezin V, Mortensen MT, Lerche MH, Jensen PH, Soroka V, Saffells JL, Doherty P, Poulsen FM, Bock E, Holm A (1999) Identification of a neuritogenic ligand of the neural cell adhesion molecule using a combinatorial library of synthetic peptides. Nat Biotechnol 17:1000–1005.

Rousselot P, Lois C, Alvarez-Buylla A (1995) Embryonic (PSA) N-CAM reveals chains of migrating neuroblasts between the lateral ventricle and the olfactory bulb of adult mice. J Comp Neurol 351:51–61.

Rutishauser U, Acheson A, Hall AK, Mann DM, Sunshine J (1988) The neural cell adhesion molecule (N-CAM) as a regulator of cell-cell interactions. Science 240:53–57.

Scheffler B, Horn M, Blumcke I, Laywell ED, Coomes D, Kukekov VG, Steindler DA (1999) Marrow-mindedness: a perspective on neuropoiesis. Trends Neurosci 22:348–357.

Schwob JE, Gottlieb DI (1988) Purification and characterization of an antigen that is spatially segregated in the primary olfactory projection. J Neurosci 8:3470–3480.

Seki T, Arai Y (1991) The persistent expression of a highly polysialylated N-CAM in the dentate gyrus of the adult rat. Neurosci Res 12:503–513.

Sporns O, Edelman GM, Crossin KL (1995) The neural cell adhesion molecule (N-CAM) inhibits proliferation in primary cultures of rat astrocytes. Proc Natl Acad Sci USA 92:542–546.

Temple S, Davis AA (1994) Isolated rat cortical progenitor cells are maintained in division in vitro by membrane-associated factors. Development 120:999–1008.

Temple S, Qian X (1995) bFGF, neurotrophins, and the control of cortical neurogenesis. Neuron 15:249–252.

Thomas LB, Gates MA, Steindler DA (1996) Young neurons from the adult subependymal zone proliferate and migrate along an astrocyte, extracellular matrix-rich pathway. Glia 17:1–14.

Tomasiewicz H, Ono K, Yee D, Thompson C, Goridis C, Rutishauser U, Magnuson T (1993) Genetic deletion of a neural cell adhesion molecule variant (N-CAM-180) produces distinct defects in the central nervous system. Neuron 11:1163–1174.

Vicario-Abejon C, Johe KK, Hazel TG, Collazo D, McKay RD (1995) Functions of basic fibroblast growth factor and neurotrophins in the differentiation of hippocampal neurons. Neuron 15:105–114.

Wade SB, Oommen P, Conner WC, Earnest DJ, Miranda RC (1999) Overlapping and divergent actions of estrogen and the neurotrophins on cell fate and p53-dependent signal transduction in conditionally immortalized cerebral cortical neuroblasts. J Neurosci 19:6994–7006.

Williams BP, Price J (1995) Evidence for multiple precursor cell types in the embryonic rat cerebral cortex. Neuron 14:1181–1188.

Williams BP, Park JK, Alberta JA, Muhlebach SG, Hwang GY, Roberts TM, Stiles CD (1997) A PDGF-regulated immediate early gene response initiates neuronal differentiation in ventricular zone progenitor cells. Neuron 18:553–562.

Williams EJ, Furness J, Walsh FS, Doherty P (1994) Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin. Neuron 13:583–594.

Winkler C, Fricker RA, Gates MA, Olsson M, Hammag JP, Carpenter M, Bjorklund A (1998) Incorporation and glial differentiation of mouse EGF-responsive neural progenitor cells after transplantation into the embryonic rat brain. Mol Cell Neurosci 11:99–116.

Yoshihara Y, Kawasaki M, Tamada A, Fujita H, Hayashi H, Kagamiyama H, Mori K (1997) OCAM: a new member of the neural cell adhesion molecule family related to zone–zone projection of olfactory and vomeronasal axons. J Neurosci 17:5830–5842.

Zigova T, Pencae V, Wiegand SJ, Luskin M (1998) Intraventricular administration of BDNF increases the number of newly generated neurons in the adult olfactory bulb. Mol Cell Neurosci 11:234–245.