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Neural cell adhesion molecule (NCAM) promotes the differentiation of hippocampal precursor cells to a neuronal lineage, especially to a glutamatergic neural cell type

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Abbreviations: bHLH, basic helix-loop-helix; BrdU, bromodeoxyuridine; CaMKII, calcium/calmodulin-dependent protein kinase II; DG, dentate gyrus; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; GluR1, AMPA receptor subunit1; MAP2, microtubule associated protein-2; MAPK, mitogen-activated protein kinase; NCAM, neural cell adhesion molecule; NeuN, neuronal nuclear protein; NR1, NMDA receptor subunit1

Abstract

Rat hippocampal precursor cells isolated from hippocampi of embryonic day 16.5 (E16.5) rat embryos were found to proliferate in the presence of basic fibroblast growth factor. Addition of soluble neural cell adhesion molecule (NCAM) to these precursor cells reduced cell proliferation in a dose dependent manner and enhanced the induction of precursor cells' differentiation to the neuronal lineage. Given these findings that NCAM induces the differentiation of hippocampal precursor cells, we investigated possible effects of NCAM on the expression of basic helix-loop-helix (bHLH) transcription factors during the differentiation. Soluble NCAM upregulated the transcription of bHLH transcription factors, neurogenin1 and NeuroD, but decreased HES5. Western blot analysis showed that NCAM increased the expression levels of CaMKII, p-MAPK, GluR1 and NR1 but decreased p-STAT3. These results support a role for NCAM in the inhibition of proliferation and the induction of neural differentiation of hippocampal neural precursor cells, and act as developmental regulators of the bHLH families, ultimately leading to the generation of glutamatergic neural cell types in the differentiation of hippocampal precursor cells.

Keywords: cell differentiation; hippocampus; neural cell adhesion molecules; neurons; rat; stem cells

Introduction

Stem cells are undifferentiated cells that display high proliferative potential, generate a wide variety of differentiated progeny including the principal phenotypes of the tissue, possess the capacity for self-renewal, and retain their multilineage potential (Gage *et al.*, 1995). Neural stem cells can generate cells of glial and neuronal lineages *in vitro* in the presence of particular neurotrophic factors (Ghosh and Greenberg, 1995; Vicario-Abejon *et al.*, 1995; Kahn *et al.*, 1997; Willians *et al.*, 1997). These cells have been the object of increasing attention for their potential use in cell replacement or gene therapy (Pincus *et al.*, 1998).

The neural cell adhesion molecule (NCAM), a member of the immunoglobulin superfamily, is widely expressed in the nervous system. NCAM is found in a variety of isoforms by alternative splicing of a single copy gene. Three major forms are NCAM 140, 180 and 120. These isoforms are structurally distinct and have different important roles in the central nervous system. NCAM mediates cell adhesion through homophilic as well as through heterophilic interactions. It 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, and also participate in neural regeneration, neurite fasciculation, and synaptogenesis in the mature nervous system (Edelman and Crossin, 1991; Lynch *et al.*, 1991; Doherty and Walsh, 1996). Polysialylation of all NCAM isoforms can occur at different times of development by the addition of a number of molecules of sialic acid. The polysialic acid NCAM (PSA-NCAM) is typically expressed during development of the nervous system, and is probably involved in neural cell migration and axonal growth (Massaro, 2002).

The involvement of neural CAMs in the control of

neural stem cell proliferation and differentiation has been studied. It was reported that NCAM binding inhibits astrocyte proliferation *in vitro* and *in vivo* after a lesion (Krushel *et al.*, 1998) and decreases the proliferation of other NCAM-expressing cell lines (Krushel *et al.*, 1998). In addition, NCAM and its ligands play a role in controlling the proliferation of neural precursor cells and directing their differentiation toward a neuronal lineage (Amoureux *et al.*, 2000). However, the involvement of NCAM in the control of neurogenic gene expression and functional properties of differentiated cells in CNS precursor cells has not been studied directly.

Several basic helix-loop-helix (bHLH) transcription factors have been implicated as mediators of neuronal or oligodendrocyte differentiation in the developing CNS. Among these bHLH factors, Mash1, NeuroD, Neurogenin promote neuronal differentiation. The proneurogenic bHLH genes include homologs of Drosophila atonal and have been divided into two groups (Kageyama *et al.*, 1997; Lee, 1997). The first group, termed neuronal determination genes, is involved in the initial commitment to a neuronal fate and includes Neurogenin1 (Ngn1) and Ngn2 (Ma *et al.*, 1996, 1997, 1998; Sommer *et al.*, 1996). The second group, termed the neuronal differentiation genes, comprises other atonal homologs including NeuroD/BETA2, NeuroD2/NDRF, Math2/Nex1, and Math3. This group is thought to regulate later events in neuronal differentiation (Bartholom and Nave, 1994; Naya *et al.*, 1995; Kageyama *et al.*, 1997; Lee, 1997; Tsuda *et al.*, 1998). Prox-1, expressed in early developing brain, encodes a homeobox protein that is structurally homologous to *Drosophila prospero* and controls early development of the central nervous system (Torii *et al.*, 1999). The expression of Mash-1 concomitantly induced expression of Prox-1 in neural stem cells of CNS (Torii *et al.*, 1999). In contrast, the mammalian homologs of the Drosophila hairy and enhancer-of-split (Hes1 and Hes5) and Notch are negative regulator of neuronal differentiation (Kageyama *et al.*, 1997). They block commitment to a neuronal fate (Ishibashi *et al.*, 1995; Tomita *et al.*, 1996; Kageyama *et al.*, 1997).

We hypothesized that NCAM may play a role in influencing the balance between proliferation and differentiation of neural stem cells and the expression of bHLH transcription factors may be altered during the differentiation of NCAM treated rat hippocampal precursor cells. The present study investigated the effect of NCAM on hippocampal precursor cell proliferation and differentiation. We show here that NCAM plays a role in controlling the proliferation of neural precursor cells and directing their differentiation to a neuronal lineage through alteration of bHLH transcription factors, signal transduction molecules and activation of functional glutamate receptors.

Materials and Methods

Rat hippocampal precursor cell culture

The conditions previously used (Johe *et al.*, 1996; Amoureux *et al.*, 2000) were adapted for this study with slight modifications, and the resulting cultures were characterized. Briefly, hippocampi were dissected from embryonic day 16.5 (E16.5) rat embryos into HBSS without calcium or magnesium. Cells obtained by mechanical dissociation was plated on 10-cm-diameter dishes coated with 15 μg/ml polyornithine and 1 μg/ml fibronectin at 2×10^6 cells per dish in Dulbecco's modified Eagle's medium (DMEM)/nutrient mixture F12 (F12)/insulin, apotransferrin, putrescine, progesterone, sodium selenite (N2 media) and incubated at 37°C in 95% O_2/5% CO_2 gas. Basic fibroblast growth factor (bFGF; 20 ng/ml; R&D system, Minneapolis, MN) was added every day in order to expand the population of proliferative precursor cells. On the confluency of 80%, these cells were subcultured once in N2 medium in the presence of bFGF at 6×10^4 per well (24-well plate) and these subcultured cells were designated as "passaged once (P1)". At this time, ~70% of the cells incorporated BrdU over 24 h and >95% of the cells expressed nestin, an intermediate filament protein specific to neural stem cells and precursors. Less than 3% of cells expressed the neuronal markers, microtubule associated protein-2 (MAP2). All experiments were done using the P1 neural precursors. The precursors were induced to be differentiated by withdrawing bFGF, and kept in a differentiation media (Neurobasal medium) for 5-7 days.

Proliferation assay

5-bromo-2'-deoxyuridine (BrdU; 10 μM) was added to the cells for 24 h before fixing. Cells were washed twice with PBS and fixed with cold 100% Methanol for 15 min on ice followed by rehydration with PBS at least 5 min. Then, cells were incubated with 2 M HCl for 45 min at room temperature, washed three times with PBS, incubated with 0.1 M sodium borate (pH 8.5) for 15 min and washed three times with PBS. Anti-BrdU antibody (Accurate Chemical, Westbury, NY) with 5% normal goat serum (NGS) was added for overnight at 4°C. Then, cells were incubated with Cy2-anti Rat IgG antibody (Jackson Laboratories) for 1.5 h at room temperature, washed twice with PBS followed by washing with distilled water, then, mounted with Vectasheild with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and analyzed under fluorescence microscope (Nikon).

Apoptosis assay

Cells were fixed with 0.15% picric acid/4% paraformaldehyde/PBS, washed twice with 0.1% BSA/PBS followed by washing with PBS and incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. Then, cells were washed twice with PBS and in situ terminal deoxynucleotidyl transferase (TdT)-mediated dUDP-biotin nick end-labeling (TUNEL) reaction mixture was added (*in situ* cell death detection, POD, Roche) and incubated in humidified chamber for 60 min at 37°C. Cells were washed three times with PBS and analyzed under a fluorescence microscope.

Immunocytochemistry

Cells were fixed with 0.15% picric acid/ 4% paraformaldehyde/PBS, washed twice with 0.1% BSA/PBS followed by washing with PBS. After washing, cells were permiabilized and blocked with 0.3% Triton-X-100/10% NGS/0.1% bovine serum albumin (BSA)/ PBS for 45 min. Primary antibody [MAP2 (Sigma, 1:200), anti-class III beta-tubulin (Tuj1, 1:200)] was applied for overnight at 4°C. Primary antibody was washed with 0.1% BSA/PBS three times and biotinylated secondary antibody (Vector Laboratories) or fluorescent labeled secondary antibody (Jackson Laboratories) was applied for 45 min-2 h. Stained cells were mounted with Vectasheild with DAPI (Vector Laboratories) and analyzed under fluorescence microscope.

Table 1. Nucleotide sequences of primers used for RT-PCR.

Genes	Orientation	Sequence
GAPDH	Sense	CTCGTCTCATAGACAAGATGGTGAAG
	Antisense	AGACTCCACGACATACTCAGCACC
HES5	Sense	AAGTACCGTGGCGGTGGAGAT
	Antisense	CGCTGGAAGTGGTAAAGCAGCTT
NeuroD	Sense	CTCAGTTCTCAGGACGAGGA
	Antisense	TAGTTCTTGGCCAAGCGCAG
Neurogenin	Sense	CCGACGACACCAAGCTCACCAAGAT
	Antisense	GTGGTATGGGATGAAACAGGGCGTC
Mash1	Sense	AGCAGCTGCTGGACGAGCA
	Antisense	CCTGCTTCCAAAGTCCATTC
Prox1	Sense	TCTTAAGCCGGCAAACCAAGAGGA
	Antisense	TTGCTCCTGGAAAAGGCATCATGG
NR1	Sense	ACACAGGAGCGGGTAAACAAC
	Antisense	CTTGTCTGAGGGGTTTCTGAG
GluR1	Sense	CCTTTGGCCTATGAGATCTGGATGTG
	Antisense	TCGTACCACCATTTGTTTTTCA

Reverse transcription (RT)-PCR

RNA was extracted with Tri reagent (Molecular Research Center) according to the manufacturer's instruction. cDNA was synthesized with 1 μg/μl RNA, 50 μM oligo(dT) at 42°C for 10 min, followed by adding superscript II, RNAsin, 2.5 mM dNTP at 70°C for 1 h. PCR was performed with the synthesized cDNA, 2.5 mM dNTP and 50 pmole primers (Table 1).

Western blotting

Protein extracts were mixed with SDS sample buffer (62.5 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% bromophenol blue) and boiled for 5 min for denaturation. Extracts were electrophoresed on 10% polyacrylamide gels and transferred onto nitrocellulose filters (Amersham Pharmacia Biotech.). Blots were blocked with 5% non-fat milk in buffer containing TBST (10 mM Tris pH 7.4, 150 mM NaCl, and 0.1% Tween-20) for 1 h at room temperature and incubated overnight at 4°C with anti-p-MAPK (1:2000, monoclonal, Cell Signaling), anti-MAPK (1:1000, polyclonal, Cell Signaling), anti-NR1 (1 μg/ml, polyclonal, Upstate Biotechnology), anti-GluR1 (1 μg/ml, polyclonal, Upstate biotechnology), anti-p-STAT3 (1:1000, polyclonal, Cell Signaling), anti-STAT3 (1:1000, polyclonal, Cell Signaling), CaMKII (1:100; ABR), and p-CaMKII (1:50; ABR) in TBST with 5% non-fat milk. After washing three times with TBST, the membranes were incubated with anti-rabbit or anti-mouse horseradish peroxidase-linked secondary antibody (1:2000; New England Biolabs, MA) for 1.5 h at room temperature. Membranes were washed three times with TBST and processed for chemiluminescence detection using ECL detection system (Amersham Pharmacia Biotech). The intensity of signals was exposed on BioMax MR-1 film (Sigma) and developed.

Results

NCAM promotes the differentiation of rat hippocampal precursor cells

We hypothesized that NCAM may influence the balance between the proliferation and the differentiation of neural precursor cells. To address this possibility, P1 precursors were expanded in N2 in the presence of bFGF, were rinsed free of bFGF and expanded in new N2 medium. Then, soluble NCAM was added for 3 days in the absence of bFGF to allow NCAM-generated cell differentiation. We treated soluble NCAM at various concentrations (1.4, 4.2, 7 and 9.8 μg/ml), followed by double immunocytochemistry for MAP2/ GFAP (Figure 1A). Cells of the neuronal lineage were identified by MAP2 expression, and cells of the astrocyte lineage were identified by GFAP expression.

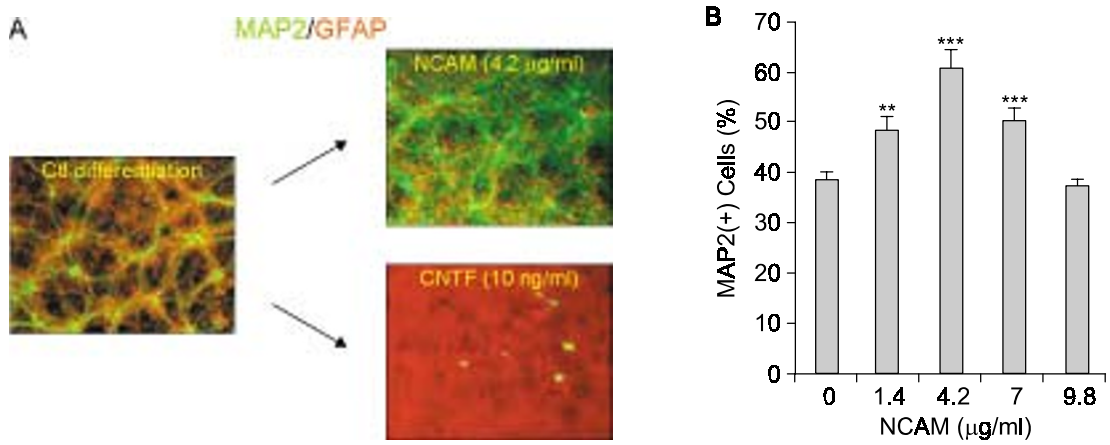

Figure 1. Stimulation of differentiation of rat hippocampal neural precursor cells toward a neuronal phenotype by NCAM. Cells were grown for 3 days on poly-L-ornithine- and fibronectin-coated dishes in N2 media in the presence of bFGF alone (20 ng/ml) and then treated for 3 days with NCAM (4.2 μg/ml) or left untreated in NB/B27 medium. Double immunocytochemical labeling for MAP2 (DTAF) and GFAP (Cy3) showing NCAM induction of MAP2(+) cells and CNTF induction of GFAP(+) cells in rat hippocampal precursor cell cultures after treatment for 3 days with either NCAM or CNTF (10 ng/ml). Cells were counterstained with DAPI (A). MAP2(+) cells and GFAP(+) cells are shown as a percentage of the total number of cells (B). The values are expressed as the average ± SEM from a representative experiment (*$P<0.05$, **$P<0.01$, ***$P<0.001$; Student's *t* test).

When NCAM was added to rat hippocampal precursor cells at the concentration of 4.2 μg/ml, a maximal increase of MAP2(+) cells was observed compared to untreated control cells (Figure 1B, untreated, 38.4±1.7%; NCAM-treated, 60.4±4.2%, $P<0.001$). On the other hand, rat hippocampal precursor cells treated with CNTF (10 ng/ml) for 3 days, cells differentiated to astrocytes expressing GFAP ($n=20$) (Figure 1A). NCAM did also significantly affect the number of astrocytes in the rat hippocampal precursor cell cultures (Figure 1A).

NCAM inhibits the proliferation of rat hippocampal precursor cells

Possible participation of NCAM on the proliferation of the neural precursor cells was examined using cells after 4 days *in vitro* (DIV) after passage. Hippocampal precursors in the presence of bFGF yielded an average 49.8±3.2% of cells BrdU-incorporated over 24 h. Whereas, cells treated with NCAM by daily addition at a dose of 7 μg/ml to cultures in the presence of bFGF, resulted in a significantly lower number of BrdU(+) cells (24.2±2.4% of cells) compared with

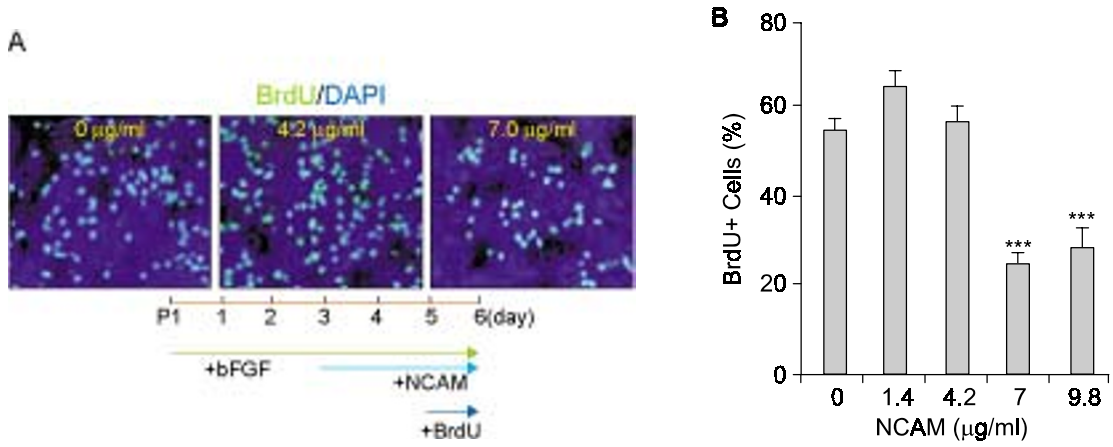

Figure 2. Inhibition of rat hippocampal precursor cell proliferation by NCAM. (A) Immunocytochemistry for BrdU incorporation in the absence (left) or presence (right) of NCAM. (B) Dose response of the effect of NCAM on rat hippocampal precursor cell proliferation measured by BrdU incorporation stimulated by 20 ng/ml bFGF. The values indicated on the graph represent the number of BrdU(+) cells as a percentage of the total number of cells (B). Values represent the average ± SEM of a minimum of six experiments (*$P<0.05$, **$P<0.01$, ***$P<0.001$; Student's *t* test).

control (Figure 2B; $P<0.001$). In the absence of bFGF, BrdU incorporation was very low after NCAM treatment for 2 days and then unaffected by NCAM treatment (data not shown). These findings suggest that NCAM support neuronal differentiation and decrease cell proliferation.

Absence of apoptotic cell death after NCAM treatment

One of the possible explanations for the decrease in BrdU incorporation may due to NCAM induced cell death. Apoptosis measured by TUNEL labeling with fluorescent nucleotides with or without NCAM treatment for 3 days is shown in Figure 3A. The percentage of TUNEL-positive cells measured as a fraction of the total cells revealed by the DAPI nuclear stain showed that few cells underwent apoptosis in either condition: 5.4±0.8% (n = 13) for untreated and 7.18±1.25% (n = 13) for NCAM-treated cultures (4.2 μg/ml) (Figure 3B, $P>0.05$). Therefore, the decrease in BrdU incorporation in the presence of NCAM is not due an increase in cell death.

Expression of several genes including bHLH transcription factors and functional glutamate receptors during differentiation of NCAM treated rat hippocampal precursor cells

In addition to the immunocytochemical results, we analyzed the temporal expression patterns of bHLH genes, and functional glutamate receptors from rat hippocampal precursor cells differentiated in the presence or absence of soluble NCAM. Semiquantitative reverse transcription-PCR was used to assay cultures at various time points for differential expression of genes regulated by NCAM or CNTF.

Neurogenin1 (Ngn1) was expressed at early stage in neuronal differentiation. As shown in Figure 4B, RT-PCR showed that its expression in control cultures was increased at 3 days (Figure 4B, 0.74±0.13, $P>0.05$) but decreased at 5 days of differentiation compared with proliferating cultures (Figure 4B, 0.49±0.07, $P>0.05$). However, in NCAM-treated cultures, the expression level of Ngn1 mRNA was significantly increased at 3 days (1.44±0.20, $P<0.001$) and 5 days (1.05±0.13, $P<0.001$) of differentiation compared with the day-matching untreated control cultures.

Similar to Ngn1, the expression level of NeuroD mRNA was significantly increased at 3 days (control, 0.4 ±0.1; NCAM, 1.2±0.3, $P<0.001$) and 5 days (control, 0.60±0.14; NCAM, 1.39±0.3, $P<0.001$) of differentiation compared with that of control cultures, whereas it was hardly increased by CNTF compared with those of control cultures (Figure 4B, CNTF: 3 days, 0.5±0.1; 5 days, 0.55±0.06, $P>0.05$). *Drosophila hairy* and *enhancer-of-split* (Hes) is widely known as anti-neurogenic member. The Hes genes block commitment to a neuronal fate (Ishibashi *et al.*, 1995; Tomita *et al.*, 1996; Kageyama *et al.*, 1997). Thus, we tested whether the expression pattern of Hes5 was changed in NCAM-treated cultures. Hes5 mRNA was largely expressed during expansion (Figure 4A). The expression level of Hes5 mRNA was not significantly changed by NCAM treatment compared with untreated control cultures (Figure 4B, 3 days:

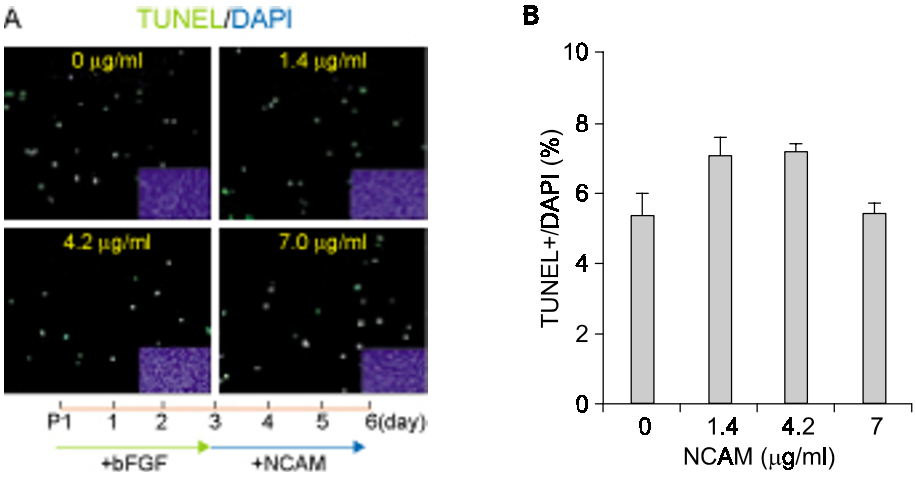

Figure 3. Absence of apoptotic cell death after NCAM treatment. (A) Hippocampal precursor cells were plated in N2 with 20 ng/ml bFGF for 72 h and treated for 3 additional days with 1.4, 4.2, 7 and 9.8 μg/ml NCAM. Apoptosis was assessed using the TUNEL method. Green fluorescent nuclei correspond to apoptotic cells. All the cells were counterstained with the nuclear stain DAPI shown in blue (*inset*). (B) The values indicated on the graph represent the number of TUNEL(+) cells as a percentage of the total number of cells. Values represent the average ± SEM of a minimum of three experiments.

control, 1.33±0.23, NCAM, 1.31±0.2, $P>0.05$; 5 days: control, 1.47±0.08, NCAM, 0.64±0.08, $P>0.05$). However, it was decreased after 3 days of differentiation in the presence of NCAM (expansion, 1.95±0.20; NCAM, 1.31±0.27, $P<0.05$) and even further decreased after 5 days of differentiation (expansion, 1.95±0.20; NCAM, 0.64±0.08, $P<0.05$) compared with expansion period.

The pro-neurogenic bHLH protein Mash1 was not affected both at 3 days (Figure 4B, control, 1.28±0.02; NCAM, 1.43±0.23, $P>0.05$) and 5 days (control, 1.8±0.2; NCAM, 1.89±0.23, $P>0.05$) of differentiation compared with the day-matching untreated control cultures. No significant differences were observed between NCAM treated cultures and CNTF treated cultures as well (Figure 4B, at 3 days, 1.35±0.22, $P>0.05$; at 5 days, 1.57±0.31, $P>0.05$). The expression pattern of Prox1 mRNA was similar to that of Mash1. Prox1 was not significantly increased both at 3 days and 5 days of differentiation compared to the day-matching untreated control cultures (Figure 4B, at 3 days: control, 0.61±0.05, NCAM, 1.71±0.1, $P>0.05$; at 5 days: control, 1.1±0.2, NCAM, 1.2±0.2, $P>0.05$).

AMPA- and NMDA-type glutamate receptors are ligand-gated ion channels that play a critical role in mediating excitatory neurotransmission and synaptic plasticity. The glutamatergic transmission is suggested to be involved in development, learning and memory (Dingledine *et al.*, 1999). To provide evidence for functional glutamate receptors activation, we undertook RT-PCR of NMDA receptor subunit NR1 and AMPA receptor subunit GluR1. Transcript levels of GluR1 and NR1, major glutamate receptor types of hippocampal neurons were significantly upregulated in NCAM treated cultures. AMPA receptor subunit 1 (GluR1) mRNA was significantly increased both at 3 days (control, 1.26±0.20; NCAM, 2.01±0.27, $P<0.01$) and 5 days (control, 1.35±0.28; NCAM, 2.17±0.28, $P<0.001$) of differentiation in NCAM treated cultures compared with the untreated control cultures (Figure 4B). The expression of NR-1 mRNA was also significantly increased both at 3 days (control, 1.65±0.18; NCAM, 2.47±0.29, $P<0.05$) and 5 days (control, 1.81±0.25; NCAM, 2.64±0.22, $P<0.01$) of differentiation in NCAM treated cultures compared to the untreated control cultures (Figure 4B). Together the experiments on immunocytochemical staining indicate that N-CAM increases the differentiation of precursor cells to a neuronal lineage.

Protein levels of signal transduction related to molecules and functional glutamate receptors were changed by NCAM

Activation of some signal transduction molecules that are known to be important for hippocampal neuronal functions such as MAPK, STAT3 and CaMKII may occur. NCAM are implicated in the control of axonal growth and the signaling potential of NCAM in neurons involves CaMKII and MAPK (Doherty *et al.*, 2000). Given the facts that NCAM acts via those prominent enzymes, we investigated the possibility that the effect of NCAM on the differentiation of hippocampal precursor cells may involve the activation of CaMKII and MAPK. The expression level of CaMKII was significantly increased in NCAM treated cultures both at 3 days (Figure 5B, control, 0.35±0.06; NCAM, 0.75±0.18, $P<0.05$) and 5 days of differentiation (control, 0.38±0.03; NCAM, 0.83±0.21, $P<0.01$). Immunoreactivity of p-CaMKII was not detected probably due to the insufficient amount. The expression level of p-MAPK level was maximum at control 3 days of differentiation, and decreased at 5 days of differentiation (Figure 5A). Whereas, in NCAM treated cultures, p-MAPK level was upregulated by 2 folds compared to control cultures at 3 days of differentiation (control, 1.01±0.04, NCAM-treated, 1.88±0.30, $P<0.05$). The increased level of p-MAPK stayed until 5 days of differentiation (control, 0.73±0.07, NCAM-treated, 2.10±0.26, $P<0.001$). CNTF causes embryonic and adult neuronal precursor cells to differentiate into astrocytes (Johe *et al.*, 1996). The CNTFR complex contains a unique CNTF-α receptor (CNTFα) component. The CNTF-induced differentiation along the glial lineage in embryonic precursor cells is mediated by activation of JAK1, STAT1, and STAT3. (Bonni *et al.*, 1997; Rajan and McKay, 1998). The expression level of p-STAT3 was increased by 2 folds in CNTF treated cultures at 3 days (control, 1.09±0.24; CNTF-treated, 1.83±0.20, $P<0.001$) and 5 days (control, 1.07±0.1; CNTF, 1.78±0.25, $P<0.001$) of differentiation compared to the day-matching untreated control cultures.

Consistent with the transcript levels of GluR1 mRNA as seen in Figure 4B, the protein levels of GluR1 was upregulated in NCAM treated cultures compared to control cultures (Figure 5B; 3 days, control, 0.35±0.03, NCAM, 0.86±0.12, $P<0.001$; 5 days, control, 0.55±0.05, NCAM, 0.95±0.09, $P<0.01$). The protein level of NR1 was upregulated in NCAM treated cultures compared to control cultures as well (Figure 5B; 3 days, control, 0.49±0.04, NCAM, 0.89± 0.06, $P<0.001$; 5 days, control, 0.57±0.09, NCAM, 1.10±0.55, $P<0.001$). Expression of the γ-aminobutyric acid (GABA)-synthesizing enzyme, glutamic acid decarboxylase (GAD), was investigated to see the effects of NCAM on the differentiation of GABAergic interneurons. The protein level of GAD67 was not changed by NCAM at 3 days (control, 0.50±0.03; NCAM, 0.59±0.05, $P>0.05$) of differentiation but decreased at 5 days (control, 0.58

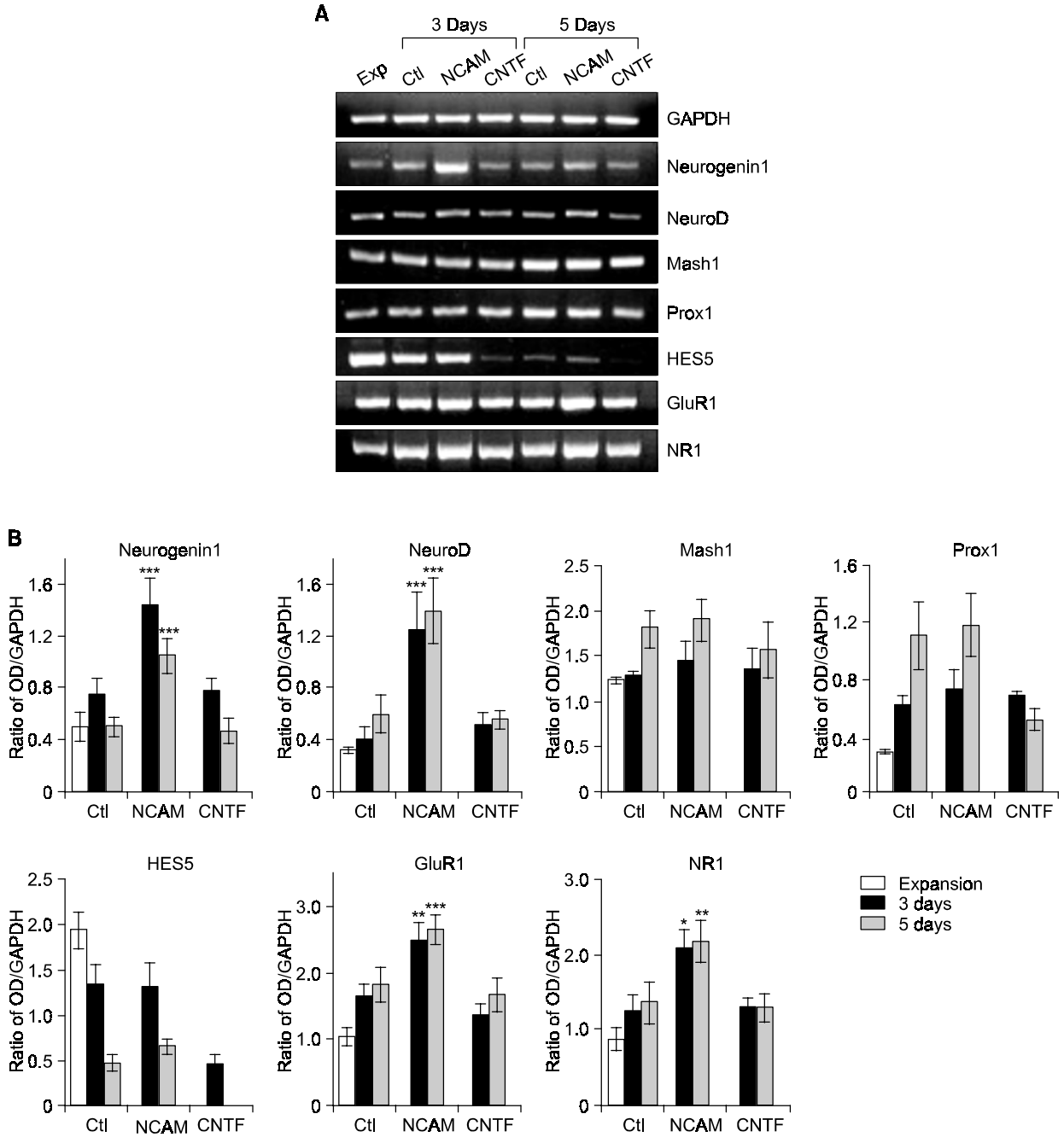

Figure 4. Molecular characterization during differentiation of rat hippocampal precursor cells. (A) Hippocampal precursor cells (P1) were treated for 3 and 5 days with either NCAM (4.2 μg/ml) or CNTF (10 ng/ml). Semiquantitative RT-PCR demonstrates differential gene expression in rat hippocampal precursor cells during differentiation. Expression of genes involved in the neural development (Neurogenin1, NeuroD, Hes5, Mash1, and Prox1) and glutamatergic neuronal receptor types (GluR1 and NR1) were assessed after 3 and 5 days of differentiation, respectively, in the absence of NCAM. A significant increase in Neurogenin1, NR1, GluR1 expression was detected during NCAM-induced differentiation (NC, NCAM; CN, CNTF). (B) Results are calculated as ratio of optical density values relative to GAPDH within each stimulus. Data are represented as mean ± SEM values ($n = 5$) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Student's t test).

± 0.04; NCAM, 0.11 ± 0.02, $P < 0.01$) of differentiation compared with the day-matching untreated control cultures. These data support the involvement of NCAM in the precursor cell differentiation toward a glutamatergic neuronal cell type.

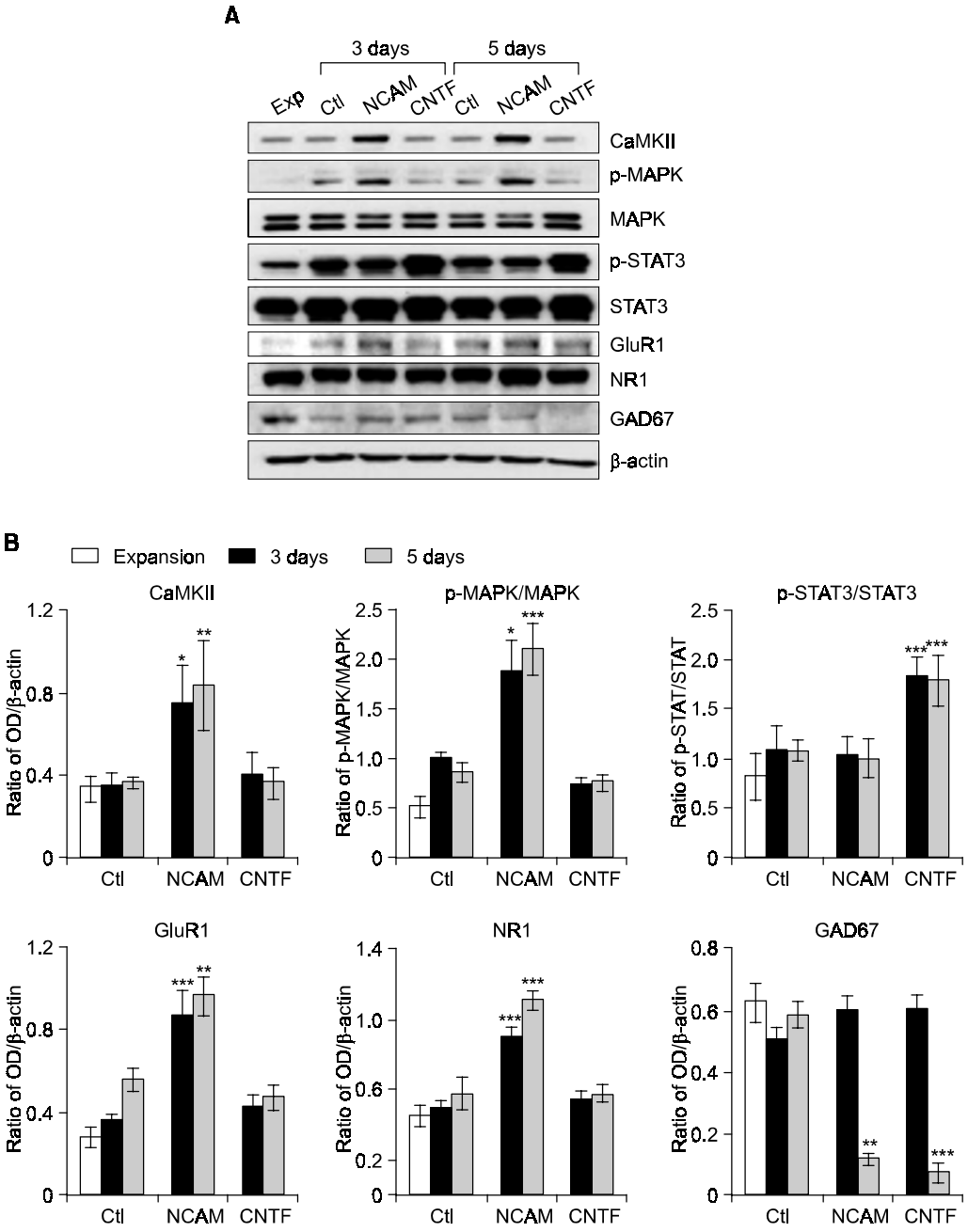

Figure 5. Increased expression of proteins involved in signal transduction and functional glutamate receptors by NCAM. (A) Hippocampal precursor cells (P1) were treated for 3-5 days with either NCAM (4.2 μg/ml) or CNTF (10 ng/ml). The protein levels of CaMKII, p-MAPK, GluR1 and NR1 were significantly increased at NCAM treated cultures. The expression level of p-STAT3 was increased at CNTF treated cultures. (B) In cases of MAPK and STAT, plots are presented as the ratio of phosphorylated enzyme relative to total enzyme. Otherwise, results are calculated as ratio of the optical density value relative to β-actin within each stimulus. Data are represented as mean ± SEM values ($n = 4$) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Student's t test).

Discussion

Earlier studies inidicated that NCAM affected the proliferation of astrocytes and differentiation in neurons (Krushel *et al.*, 1998) while whether an increase of neurogenic gene expression in precursor cells occurs after NCAM binding is not clear. We hypothesized that NCAM might have a significant effect on the expression of genes and proteins related to neural development and hippocampal neuronal functions.

The studies reported here support this idea and indicate that NCAM may acts as an endogenous regulator of precursor cell proliferation and differentiation.

Consistent with the previous results, in the presence of either bFGF, which induces proliferation of neural precursor cells, addition of NCAM decreased cell proliferation. The inhibition of proliferation by NCAM was not restricted to hippocampal precursor cells but also occurred in neonatal cerebellar rat precursor cell cultures and normal human neural precursor cells (Amoreaux *et al.*, 2000). NCAM binding to the surface of several neural cell types can therefore inhibit their proliferation.

Our results suggest that in hippocampal precursor cells, NCAM comprises a combinatorial code for the specification of neurons extending from transcription of genes for neural differentiation to expression of functional proteins required for neurons. It has been previously reported that Ngn1 not only promotes neurogenesis but also inhibits astrocyte differentiation and strongly inhibited LIF-induced (leukemia inhibitory factor) glial differentiation by cortical precursors in culture, independent of its ability to promote neuronal differentiation (Sun *et al.*, 2001). This raised a possibility that Ngn1 instructively promotes neuronal cell fate determination, as opposed to merely promoting neuronal differentiation, at the expense of gliogenesis. In our culture system, we could not observe the inhibition of glial differentiation by NCAM because glial cell population was far greater than neurons. It is not clear that NCAM specifies neuronal identity via proneural gene, Ngn1 from our results. However, it is clear that NCAM is far more effective on the expression of Ngn1 mRNA than MASH1 mRNA. It is consistent with previous results showing the identity specification function of neurogenins is more sensitive to context than is that of MASH1 (Lo *et al.*, 2002). It would be interesting to see if the blockade of the expression of Ngn1 inhibited the NCAM effects on differentiation.

Our results indicate that molecular signaling events underlying the cell-cell interaction mediated by NCAM may participate in precursor cell proliferation and differentiation. However, the precise mechanism by which NCAM modulates neural differentiation remains to be determined. NCAM has been reported to interact with the FGF receptor (Williams *et al.*, 1994) and act via homophilic binding (Soroka *et al.*, 2002). However, previous results demonstrated that neither FGF receptor binding nor homophilic binding is involved in the NCAM-induced inhibition of proliferation and induction of neural differentiation of neural precursor cells (Amoureux *et al.*, 2000). In astrocytes and neurons, NCAM apparently can signal via NCAM itself by a mechanism that is influenced by the first three Ig domains. We are currently investigating the extend of involvement of FGF receptor by using a new FGF receptor antibody, SU5402, in NCAM-induced neural precursor differentiation and the expression of genes and proteins which have been investigated in the present study. Whether the activation of MAPK is critical for the NCAM-induced differentiation of neural precursor cells is also currently investigated by using constitutive active and dominant negative MEK-expressing neural precursor cells. NCAM also has been implicated in adult hippocampal neurogenesis. The clustering and a microenvironment provided by the PSA-NCAM-expressing immature neurons may contribute to the early developmental events of adult neurogenesis, such as proliferation and differentiation in the adult hippocampus (Seki *et al.*, 2002).

In conclusion, our results suggest that NCAM induces functional properties of neurons besides the neuron subtype identities they can promote. These properties of NCAM may explain cellular contributions of NCAM in neuronal differentiation during embryonic development and adult brain.

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