EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 38, No. 6, 732-738, December 2006

Neural cell adhesion molecule (NCAM) induces neuronal phenotype acquisition in dominant negative MEK1-expressing hippocampal neural progenitor cells

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Accepted 7 November 2006

Abbreviations: bFGF, basic fibroblast growth factor; CMV, cytomegalovirus; DN, dominant negative; EGFP, enhanced green fluorescence protein; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MEK, MAP kinase kinase; NB, neurobasal; NCAM, neural cell adhesion molecule; NPCs, neural progenitor cells; Tuj1, β-tubulin type III

Abstract

It has been shown that neural cell adhesion molecule (NCAM)-induced neuronal differentiation is extracellular signal-regulated kinase (ERK)-dependent. However, an involvement of the mitogen activated protein kinase (MAPK) kinase (MEK), an upstream kinase of ERK, has not been directly demonstrated in this process. Therefore, we investigated whether the MEK1 plays a critical role in the NCAM-induced neuronal differentiation of hippocampal neural progenitor cells (NPCs). NPCs were transiently transfected with expression plasmids encoding activated or dominant negative (DN) forms of MEK1. The expression of DN MEK1 inhibited neuronal phenotype acquisition and soluble NCAM rescued the defect in the neuronal phenotype acquisition in DN-MEK1-transfected cells, suggesting that NCAM might contribute to the neuronal differentiation *via* distinct, parallel pathways including the MEK pathway. In cells expressing wild type MEK1 or constitutively active MEK1 on the other hand, the percentage of cells positive for β-tubulin type III (Tuj1), a marker for early postmitotic neurons, was higher than seen in vector-transfected cells. These results suggest that the activation of MEK1 is required for obtaining neuronal phenotype in NPCs.

Keywords: hippocampus; MAP kinase kinase 1; neural cell adhesion molecules; rats

Introduction

Neural cell adhesion molecule (NCAM) is a member of the immunoglobulin (Ig) superfamily and is involved in a variety of cellular processes of importance for the formation and maintenance of the nervous system. The role of NCAM in neural cells is presumed to depend on the modulation of intracellular signal transduction cascades. Homophilic NCAM interactions stimulate neurite outgrowth apparently through phosphorylation of the fibroblast growth factor receptor (FGF-R), which in turn activates phospholipase Cγ (PLCγ) to generate diacylglycerol (DAG) (Williams *et al*., 1994a; 1994c; Saffell *et al*., 1997) and subsequently arachidonic acid, which has been suggested to increase a voltage-dependent calcium channels (VDCC)-induced calcium influx (Williams *et al.,* 1994b; 1995). Moreover, two non-receptor tyrosine kinases, p59 ^{fyn} and the focal adhesion kinase, FAK, have been reported to interact with the 140-kDa isoform of NCAM (NCAM-140) (Beggs *et al*., 1994; 1997). In line with these findings, Kolkova *et al.* could indeed show that neurite outgrowth from PC12-E2 cells grown on substrata allowing homophilic NCAM interactions is dependent on the MAPK pathway (Kolkova *et al*., 2000).

NCAM is also involved in neuronal differentiation. We previously demonstrated that soluble NCAM increased the differentiation of progenitor cells to the neuronal lineage with a concurrent upregulation of the proneural basic helix-loop-helix (bHLH) transcription factors, neurogenin1 and NeuroD (Shin *et al*., 2002). Furthermore, overexpression of NCAM-140 facilitates hippocampal neurogenesis *via* regulation of proneurogenic transcription factors in an extracellular signal-regulated kinase (ERK)-dependent manner (Kim *et al*., 2005). Exogeneously added purified soluble NCAM is shown to induce homophilic NCAM binding (Krushel *et al*., 1998). Overexpressed NCAM-140 is expected to increase homophilic NCAM binding as well (Kim *et al*., 2005). Increase in homophilic NCAM binding either by soluble NCAM and overexpression of NCAM-140 is directly related to the activation of NCAM-dependent signaling pathways.

The aim of our study was to assess whether MEK upstream of ERK1/2, especially MEK1, is critical for NCAM-induced neuronal differentiation and neurite outgrowth. In addition to our previous results that NCAM increases the differentiation of hippocampal NPCs *via* an ERK-dependent pathway, some reports showed that MEK1 is activated during neuronal differentiation of PC12 cells upon NGF treatment (Jaisawal *et al*., 1993; Cowley *et al*., 1994). Therefore, we hypothesized that MEK1 might play a pivotal role in neuronal differentiation of NPCs *via* interaction with a NCAM-140-dependent pathway and investigated whether the expression of the dominant negative construct of MEK1 in NPCs negatively influences neuronal differentiation including neuronal fate acquisition and whether soluble NCAM is able to rescue the effects of dominant negative MEK1s. We here report that neuronal phenotype acquisition is dependent on activation of a MEK1-dependent signaling cascade. Furthermore, soluble NCAM seems to be able to stimulate neuronal differentiation through mechanisms independent of the MEK pathway.

Materials and Methods

Rat hippocampal NPC culture

Hippocampal NPC cultures were obtained as previously described in Kim *et al*. (2005), with slight modifications. Embryos from Sprague Dawley adult pregnant female rats (Harlan Sprague Dawley, Indianapolis, IN) were used. Briefly, hippocampi were dissected from embryonic day 16.5 (E16.5) rat embryos into Hank's balanced salt solution (HBSS) without calcium or magnesium. Cells were plated on 10-cm-diameter dishes coated with 15 μg/ml poly-L-ornithine and 1 μg/ml fibronectin (Invitrogen, Carlsbad, CA) at 2.5×10^4 cells/cm^2 in N2 media and incubated at 37°C in 95% air/5% CO_2 gas. Basic fibroblast growth factor (bFGF, 20 ng/ml, R&D system, Minneapolis, MN) was daily added in order to expand the population of proliferative progenitors and medium was changed every 2 d at the time of bFGF addition (Kim *et al*., 2005). The cells on 80% confluency were transfected with the following expression vectors using V-solution according to the manufacturer's protocol (Amaxa GmbH, Cologne, Germany).

Mutant MEK1 expression vectors

The DN MEK1 expression vectors (S222A and K97A) and the constitutively active MEK1 expression vectors (S222E and S218, 222E) were previously described (Seger *et al*., 1994; Alessandrini *et al*., 1996). S222A MEK1 is a mutant MEK1 in which Ser222 is mutated to Ala. K97A MEK1 is a mutant that Lys97 is mutated to Ala. S222E MEK1 corresponds to a mutant MEK1 in which Ser site is mutated to Glu. S218, 222E MEK1 corresponds to a mutant that both Ser218 and 222 are mutated to Glu. The MEK1 mutant expression vectors were a gift from Dr. JH Kim (Korea University) and each MEK1 phosphorylation site mutant gene was cut with *Sac*I/*Hin*dIII and cloned into MCS site digested with *Sac*I/*Hin*dIII in pEGFP-C2 expression vector (Clontech, Mountain View, CA) that also carries EGFP gene under the control of a CMV promoter. In the experiments with MEK mutants, the transfection was performed in the same conditions using 0.1 μg of the expression vectors or 0.05 μg of pCMV-wild type MEK1-EGFP. Transfected cells were cultured in N2 media containing bFGF at 6×10^4 cells/cm^2 in a 24 well plate for 24 h. Following transfection, the cells were differentiated for 3 d in the absence of bFGF either with or without soluble NCAM (4.2 μg/ml) in neurobasal (NB) media as previously used (Shin *et al*., 2002). Control cultures were treated with the same concentration (0.01%) of dimethyl sulfoxide (DMSO).

Immunocytochemistry and analysis

After 72 h of differentiation, cells were fixed with 0.15% picric acid/4% paraformaldehyde/PBS, washed twice with 0.1% bovine serum albumin (BSA)/PBS followed by washing with PBS. After washing, cells were permeabilized and blocked with 0.3% Triton-X-100/10% NGS/0.1% BSA/PBS for 45 min. Primary antibody [anti-Tuj1 (Covance, Berkeley, CA; 1:500) or anti-GFP (Roche, Basel, Switzerland; 1:200)] was applied for overnight at 4°C. Primary antibodies were washed with 0.1% BSA/PBS three times and then anti-Tuj1 was detected by Cy3-conjugated secondary antibody (1:100; Jackson ImmunoResearch, West Grove, PA), respectively. The anti-GFP signal was amplified by biotinlyated anti-mouse IgG and then detected by Streptavidin-conjugated fluorescein (DTAF). Stained cells were mounted with Vectashield with DAPI (Vector Laboratories) and analyzed under a fluorescence microscope (Nikon, Tokyo, Japan). All transfections were repeated at least five times using different plasmid preparations and gave similar results. EGFP(+) cells from randomly selected areas of at least four independent experiments were photographed. Morphological characteristics were quantitated using Sigmascan Pro. (SPSS, Chicago, IL). Statistical significance was determined using Student's *t* test. All statistical analyses were conducted with Sigma plot 2000 software.

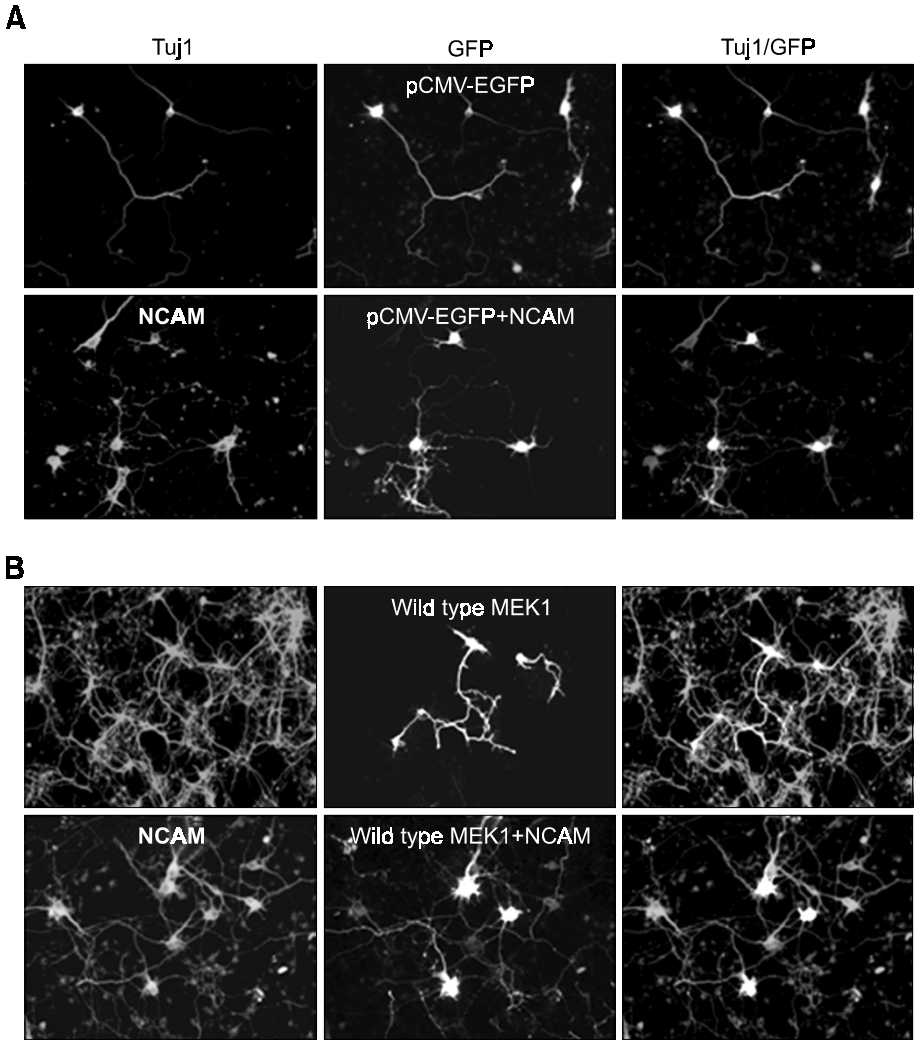


Figure 1. Effects of mutant MEK1 expression and soluble NCAM treatment on neuronal differentiation of hippocampal NPCs. Dual-label immunocytochemistry for Tuj1(+) (red) and GFP(+) (green) cells was performed 3 d after inducing differentiation. Hippocampal NPCs were transfected with pCMV-EGFP (A), pCMV-wild type MEK1-EGFP (B), pCMV-K97A MEK1-EGFP (C), pCMV-S222A MEK1-EGFP (D), pCMV-S222E MEK1-EGFP (E), or pCMV-S218,222E MEK1-EGFP (F). After transfection, cells were spread onto 24 well dishes, and cultured in N2 media in the presence of bFGF for 24 h. Then, cells were induced to differentiate by withdrawal of bFGF in the absence or presence of soluble NCAM for 3 d, followed by dual immunocytochemistry for GFP and Tuj1. Scale bar, 100 μm.

Results and Discussion

Mutant MEK1 expression in NPCs

In order to correlate MEK1 activity with neuronal differentiation, undifferentiated proliferating hippocampal NPCs were transfected with either DN mutants (S222A and K97A) or constitutively active mutants (S222E and S218, 222E) of the MEK1. Each MEK1 mutant gene was inserted into a CMV-EGFP expression vector as mentioned in materials and methods. The GFP signal was analysed 24 h after transfection and 3-5% of the NPCs expressed GFP (data not shown). The GFP-expression in transfected cells allowed us to directly characterize the general morphology and neuronal phenotype acquisition of those transfected cells.

Fourty-eight hours after transfection, the intensity of the GFP signal had reached a level similar to that shown in Figure 1 in the transfected cells. At this point the cells expressing wild type MEK1, constitutively active S222E MEK1 or S218, 222E MEK1 began to exhibit a differentiated neuron-like morphology, which reached its most distinguishable level around 3 d after inducing differentiation. Cells expressing wild type MEK1, S222E MEK1, or S218, 222E MEK1 were morphologically indistinguishable (Figure 1).

MEKs for neuronal differentiation

We next investigated whether the expression of mutant MEK1s affected neuronal differentiation. The DN-MEK1-expressing cells (Figure 1C and D) did not express Tuj1 nor exhibit neurite outgrowth compared to empty vector-transfected cells (Figure 1A) or wild type MEK1-transfected cells (Figure 1B), suggesting that DN-MEK1 is unable to induce the acquisition of the neuronal phenotype. In contrast, cells expressing the constitutively active S222E MEK1 or S218, 222E MEK1 expressed Tuj1 (Figure

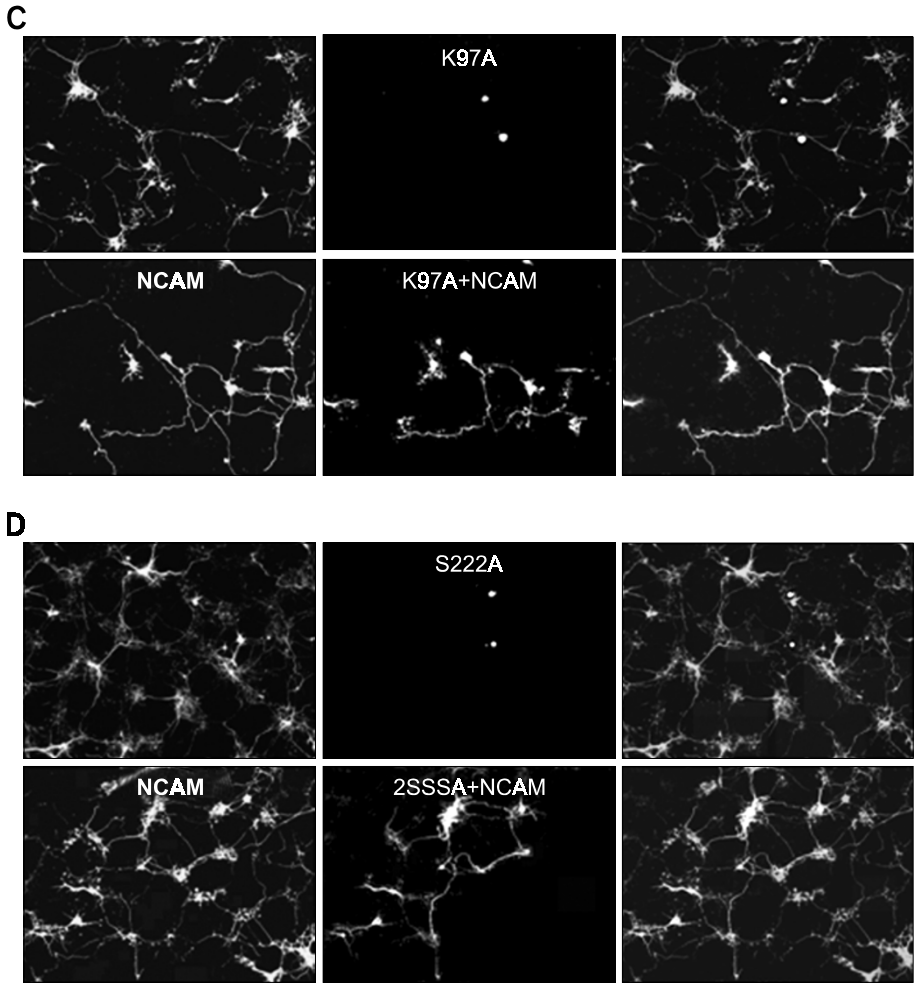
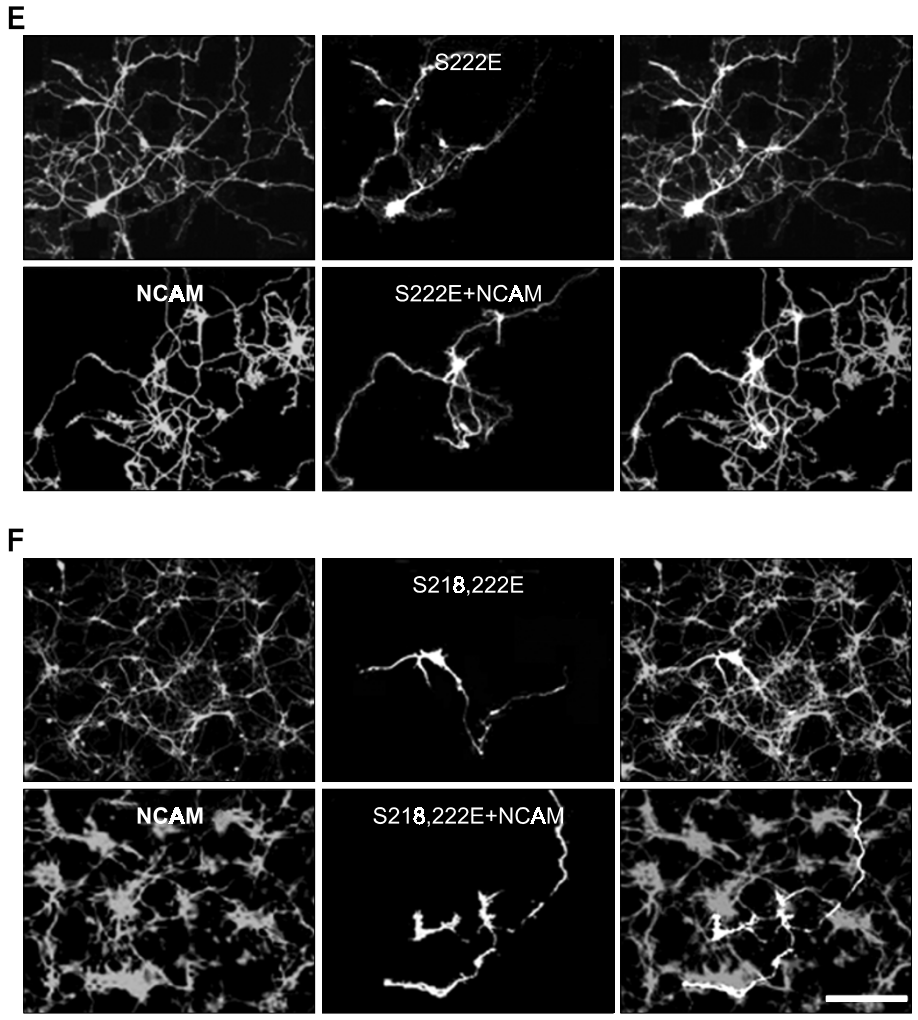


Figure 1. Continued.

1E and F) with more distinct morphological changes such as an extended neurite outgrowth with local axonal arborizations, indicating that MEK1 activity is involved in the neuronal phenotype acquisition, which is consistent with previous findings (Leppa *et al*., 1998; Kolkova *et al*., 2000). When the fraction of Tuj1(+) neurons among the transfected GFP(+) cells was compared between cells expressing DN-MEK1 and constitutively active MEK1, DN-MEK1 induced neuronal differentiation in less than 1% of transfected GFP(+) cells (Figure 1C, D and Figure 2). Whereas the expression of constitutively active MEK1 induced neuronal differentiation in 40% of transfected cells (Figure 1E, F and Figure 2). Thus the capability of expressing Tuj1 seems to correlate closely with MEK1 activation.

Actions of soluble NCAM in dominant negative MEK1-transfected NPCs

We previously demonstrated that addition of soluble NCAM to hippocampal NPCs reduced cell proliferation and increased neuronal differentiation with concurrent upregulation of the proneural bHLH transcription factors, neurogenin1 and NeuroD (Shin *et al*., 2002). To investigate whether the effects of DN-MEK1 on the neuronal phenotype acquisition were altered by soluble NCAM treatment, we examined the fraction of Tuj1(+) cells in response to soluble NCAM treatment in the DN-MEK1 transfected cells. In a control experiment investigating the effects of soluble NCAM, cells transfected with wild type MEK1 were included. An increased fraction of Tuj1(+) cells was detected 3 d after daily treatment with soluble NCAM in wild type MEK1-transfected cells (Figure 1B and Figure 2). A relatively high increase in the fraction of Tuj1(+) cells was detected in DN-MEK1 transfected cells (Figure 1C, D and Figure 2) after soluble NCAM treatment. Compared to the DN-MEK1 transfected cells, a further increase in the fraction of Tuj1(+) cells was not observed in the constitutively active MEK1-transfected cells treated with soluble NCAM (Figure 1E, F and Figure 2), demonstrating that neuronal differentiation might



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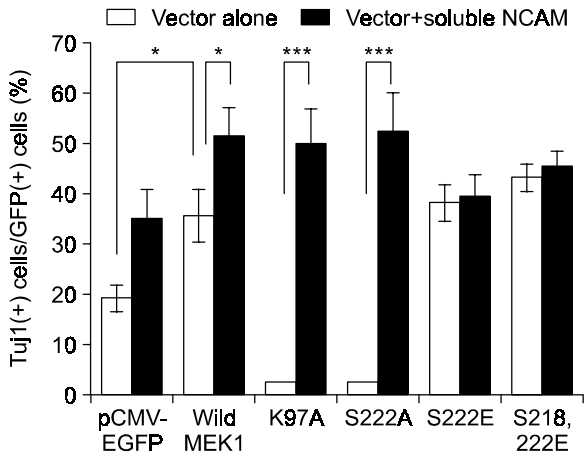

Figure 2. Effects of mutant MEK1 expression on neuronal phenotype acquisition. Tuj1(+) cells were counted and expressed as the percentage change with respect to GFP(+) cells. Each value represents the average ± SEM of five independent experiments ($n = 5$). Data are presented as means ± SEM. Student's *t*-test: $^{*}P < 0.05$, $^{***}P < 0.001$.

be fully achieved *via* a MEK-dependent pathway. Taken together, our results directly demonstrate that a MEK-dependent pathway plays a role in neuronal phenotype acquisition and the defect in neuronal phenotype acquisition caused by DN-MEK was, at least partially, rescued by soluble NCAM. Concurrently, the fact that soluble NCAM is able to partially rescue neuronal phenotype acquisition in DN-MEK1 transfected cells implies that soluble NCAM treatment might be able to induce the activation of a MEK1-independent signaling cascade resulting in neuronal differentiation. Supporting this possibility, our previous results showed that soluble NCAM increased the expression of CaMKII (Shin *et al.*, 2002). However, it can not be ruled out that some wild type MEK1 is activated in DN-MEK1 transfected cells and then contributes to the increase in the fraction of Tuj1(+) cells in the presence of soluble NCAM.

MEK1 activity and neuronal differentiation

There is no striking difference between wild type MEK1-transfected cells and constitutively active MEK1-transfected cells 3 d after differentiation (Figure 1B, E and F), although there is a significant difference in the fraction of Tuj1(+) cells between control empty vector- and wild type MEK1- or constitutively active MEK1-transfected cells (Figure 2). The rare difference in phenotype of the constitutively active MEK1-transfected cells and the wild type MEK1-transfected cells might suggest that the steady-state system for maintaining neuronal differentiation and the neuronal differentiation involves more than only MEK1 activity.

Because MAP kinases are direct downstream targets of MEK1, the activation of MAP kinases would be expected in cells expressing constitutively active MEK1 mutants. Although we did not directly assess whether the expression of mutant MEKs correlated with Erk activity, it is likely that the effects of the MEK1 phosphorylation site mutants reflect a differential activity of MEK1 donwstream kinases based on previous investigations (Alessandrini *et al*., 1996; Karpova *et al*., 1997; Shalin *et al*., 2004). It is not clear, however, whether the activity of other kinases is changed in DN-MEK1 or constitutively active MEK1-transfected cells, since previous investigations showed that Raf-1 kinase activity is elevated in the [Asp^{218}]MEK1-transformed cells (Alessandrini *et al*., 1996). Whether other regulators of MAP kinase activity are involved in the hippocampal NPCs remains to be determined.

It has been suggested that the various MEK1 phosphorylation mutants may form complexes with distinct proteins and/or may localize to different parts of the cell where they would not normally be present (Bottorff *et al*., 1995; Jaaro *et al*., 1997; Vaidya *et al*., 2005). The elucidation of other pathway(s) involved in MEK1 activation will shed further light on our understanding of NCAM-mediated neuronal cell differentiation.

Acknowledgement

This research was supported by a grant (M103KV-010008-06K2201-00810) from the Brain Research Center (BRC) of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, the Republic of Korea, and the research fund of Hanyang University (HY-2005-000-0000-1792) awarded to H. Son.

References

Alessandrini A, Greulich H, Huang W, Erikson RL. Mek1 phosphorylation site mutants activate Raf-1 in NIH 3T3 cells. J Biol Chem 1996;271:31612-8

Beggs HE, Soriano P, Maness PF. NCAM-dependent neurite outgrowth is inhibited in neurons from Fyn-minus mice. J Cell Biol 1994;127:825-33

Beggs HE, Baragona SC, Hemperly JJ, Maness PF. NCAM140 interacts with the focal adhesion kinase p125(fak) and the SRC-related tyrosine kinase p59(fyn). J Biol Chem 1997;272:8310-19

Bottorff D, Stang S, Agellon S, Stone JC. RAS signalling is abnormal in a c-raf1 MEK1 double mutant. Mol Cell Biol 1995;15:5113-22

Cowley S, Paterson H, Kemp P, Marshall CJ. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH3T3 cells. Cell 1994;77:841-52

Jaaro H, Rubinfeld H, Hanoch T, Seger R. Nuclear translocation of mitogen-activated protein kinase kinase (MEK1) in response to mitogenic stimulation. Proc Natl Acad Sci USA 1997;94:3742-7

Jaisawal RK, Murphy MB, Landreth GE. Identification and characterization of a nerve growth factor stimulated mitogen-activated protein kinase activator in PC12 cells. J Biol Chem 1993;268:7055-63

Karpova AY, Abe MK, Li J, Liu PT, Rhee JM, Kuo WL, Hershenson MB. MEK1 is required for PDGF-induced ERK activation and DNA synthesis in tracheal myocytes. Am J Physiol 1997;272(3 Pt 1):L558-65

Kim JH, Lee JH, Park JY, Park CH, Yun CO, Lee SH, Lee YS, Son H. Retrovirally transduced NCAM140 facilitates neuronal fate choice of hippocampal progenitor cells. J Neurochem 2005;94:417-24

Kolkova K, Novitskaya V, Pedersen N, Berezin V, Bock E. Neural cell adhesion molecule-stimulated neurite outgrowth depends on activation of protein kinase C and the Ras-mitogen-activated protein kinase pathway. J Neurosci 2000; 20:2238-46

Krushel LA, Tai MH, Cunningham BA, Edelman GM, Crossin KL. Neural cell adhesion molecule (N-CAM) domains and intracellular signaling pathways involved in the inhibition of astrocyte proliferation. Proc Natl Acad Sci USA 1998;95: 2592-6

Leppa S, Saffrich R, Ansorge W, Bohmann D. Differential regulation of c-Jun by ERK and JNK during PC12 cell differentiation. EMBO J 1998;17:4404-13

Saffell JL, Williams EJ, Mason IJ, Walsh FS, Doherty P. Expression of a dominant negative FGF receptor inhibits axonal growth and FGF receptor phosphorylation stimulated by CAMs. Neuron 1997;18:231-42

Seger R, Seger D, Reszka AA, Munar ES, Eldar-Finkelman H, Dobrowolska G, Jensen AM, Campbell JS, Fischer EH, Krebs EG. Overexpression of mitogen-activated protein kinase kinase (MAPKK) and its mutants in NIH 3T3 cells. Evidence that MAPKK involvement in cellular proliferation is regulated by phosphorylation of serine residues in its kinase subdomains VII and VIII. J Biol Chem 1994;269:25699-709

Shalin SC, Zirrgiebel U, Honsa KJ, Julien JP, Miller FD, Kaplan DR, Sweatt JD. Neuronal MEK is important for normal fear conditioning in mice. J Neurosci Res 2004;75:760-70

Shin MH, Lee EG, Lee SH, Lee YS, Son H. Neural cell adhesion molecule (NCAM) promotes the differentiation of hippocampal precursor cells to a neuronal lineage, especially to a glutamatergic neural cell type. Exp Mol Med 2002;34:401-10

Vaidya RJ, Ray RM, Johnsonk LR. MEK1 restores migration of polyamine-depleted cells by retention and activation of Rac1 in the cytoplasm. Am J Physiol Cell Physiol 2005;288: C350-9

Williams EJ, Furness J, Walsh FS, Doherty P. Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin. Neuron 1994a;13:583-94

Williams EJ, Walsh FS, Doherty P. The production of arachidonic acid can account for calcium channel activation in the second messenger pathway underlying neurite outgrowth stimulated by NCAM, N-cadherin, and L1. J Neurochem 1994b;62:1231-4

Williams EJ, Walsh FS, Doherty P. Tyrosine kinase inhibitors can differentially inhibit integrin-dependent and CAM-stimulated neurite outgrowth. J Cell Biol 1994c;124:1029-37

Williams EJ, Mittal B, Walsh FS, Doherty P. A Ca^{2+}/calmodulin kinase inhibitor, KN-62, inhibits neurite outgrowth stimulated by CAMs and FGF. Mol Cell Neurosci 1995;6:69-79