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MAPK-upstream protein kinase (MUK) regulates the radial migration of immature neurons in telencephalon of mouse embryo

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SUMMARY

The radial migration of differentiating neurons provides an essential step in the generation of laminated neocortex, although its molecular mechanism is not fully understood. We show that the protein levels of a JNK activator kinase, MUK/DLK/ZPK, and JNK activity increase potently and temporally in newly generated neurons in developing mouse telencephalon during radial migration. The ectopic expression of MUK/DLK/ZPK in neural precursor cells in utero impairs radial migration, whereas it allows these cells to leave the ventricular zone and differentiate into neural cells. The MUK/DLK/ZPK protein is associated with dotted structures that are frequently located along microtubules and with Golgi apparatus in cultured embryonic cortical cells. In COS-1 cells, MUK/DLK/ZPK overexpression impairs the radial organization of microtubules without massive depolymerization. These results suggest that MUK/DLK/ZPK and JNK regulate radial cell migration via microtubule-based events.

Key words: Cortex, JNK, Microtubules, Mouse

INTRODUCTION

The generation of the laminated neocortex depends on the radial and tangential migration of neurons newly generated from neural stem cells at the ventricular zone of the developing telencephalon (Anevine et al., 1961; Marin-Padilla et al., 1978; Rakic, 1990; Chenn and McConnell, 1995; Takahashi et al., 1996; Hatten, 1999; Parnavelas, 2000). Radial migration is guided by radial glial scaffolds, which extend from the ventricular zone to the marginal zone, and by extracellular proteins such as Reelin (D'Arcangelo et al., 1995). More recently, VLDLR/ApoER2 (Trommsdorff et al., 1999), Dab1 (Sheldon et al., 1997; Howell et al., 1997), Cdk5 (Ohshima et al., 1996) and p35 Cdk5 activator (Kwon and Tsai, 1998) have also been shown to be indispensable for the generation of laminated neocortex, and to serve as receptors for Reelin and modulators of the intracellular signal transduction that seems to act downstream of the receptor. The identification of human genes responsible for brain malformations, including type 1 lissencephalies and periventricular heterotopia, has further increased the understanding of the molecular components that regulate neural cell migration. These involve Lis1 (Reiner et al., 1993) and doublecortin (Des Portes et al., 1998), which are modulators of microtubule organization, and filamin (Fox et al., 1998), which is a modulator of microfilament organization. Despite these significant advances in the discovery of molecules responsible for neural cell migration and understanding their primary functions, the molecular mechanism that regulates cell migration remains mostly unknown.

Jun N-terminal kinases (JNKs), also known as stress-activated protein kinases (SAPKs), are members of the mitogen-activated protein kinase (MAPK) family (Kyriakis and Avruch, 2001). Double knockout of the *Jnk1* and *Jnk2* genes impairs neural tube closure and cortical development in the brain of mouse embryos, as well as regional specific apoptosis of undifferentiated neural stem cells (Kuan et al., 1999; Sabapathy et al., 1999), although the molecular mechanisms that cause these defects remain to be explored. JNK activity is regulated by protein kinases of the MAPK kinase kinase (MAPKKK) class through protein kinases of the MAPK kinase (MAPKK) class (Nishida and Gotoh, 1993; Widmann et al., 1999). We have identified a protein kinase related to mixed lineage kinases, MUK (also known as DLK and ZPK), as a MAPKKK of JNK (Hirai et al., 1996) that is highly expressed in adult and embryonic neural tissues (Holzman et al., 1994; Nadeau et al., 1997). MUK/DLK/ZPK, as well as other mixed lineage kinases have been shown to associate with scaffold proteins, JIPs, which also interact directly with MKK7, a MAPKK class protein kinase, and JNK (Whitmarsh et al., 1998; Ito et al., 1999). More recently, JIPs have been identified as cargo of the kinesin motor functioning in vesicle transport (Bowman et al., 2000; Verhey et al., 2001). In addition, JIPs carrying MUK/DLK/ZPK also bind to a Reelin receptor, ApoER2 (Stockinger et al., 2000; Verhey et al., 2001). Taken together, these observations suggest that the MUK/DLK/ZPK-JNK pathway could be involved in the regulation of cellular events supported by kinesin motors and/or Reelin signaling, such as

axonal transport and neural cell migration, although this possibility has not been tested.

To explore the significance of the MUK/DLK/ZPK-JNK signaling pathway in neural cell migration in vivo, we first examined the expression of the MUK/DLK/ZPK protein and the distribution of active JNK in developing mouse brain at different embryonic stages and found a temporal induction of MUK/DLK/ZPK expression and JNK activation in immature neurons. Then we monitored the effect of the constitutive activation of the MUK/DLK/ZPK-JNK pathway on neural migration and differentiation in utero, and found an inhibitory effect of MUK/DLK/ZPK expression on the radial migration of immature neurons. We also found that MUK/DLK/ZPK is associated with dotted structures located along microtubules as well as in Golgi apparatus in primary culture cells of E16 embryonic cortex, and that MUK/DLK/ZPK overexpression in COS1 cells alters microtubule organization. These results strongly suggest that the MUK/DLK/ZPK-JNK pathway contributes to the regulation of neural cell migration.

MATERIALS AND METHODS

Expression vectors

Plasmid vectors for rat MUK and His/T7-tagged MUK expression in mammalian cells have been described elsewhere (Hirai et al., 1996). T7-MUK contains a His/T7-tag at the N terminus of MUK that is derived from pBlueBacHisC. MUK/KR bears a single amino acid substitution at Lys185 (to Arg), which is essential for protein kinase activity. The recombinant adenovirus vector was constructed with *Eco*T22I cut Ad5 dlX virus genome, pAxCAwt cassette bearing a CAG promoter (Cytomegarovirus enhancer + chicken β-actin promoter), and an *Nhe*I-*Ssp*I fragment of T7-MUK or T7-MUK/KR plasmid vector by the COS-TPC method (Miyake et al., l996). In these constructs, the His-tag was removed and only the T7-tag remained at the N-terminus of the proteins. The recombinant adenovirus vector for *lacZ* expression, pAxCA-*lacZ*, was provided by RIKEN BRC DNA bank. The virus was amplified in 293 cells and purified by CsCl density-gradient centrifugation and dialysis against PBS.

Injection of vectors into embryonic brain

The ICR mice used in these experiments were maintained according to protocols approved by the Institutional Animal Care and Use Committee at Yokohama City University School of Medicine. The uterus of an E13-timed pregnant mouse was incised under a bio-safety cabinet. Adenovirus vector ($2\sim5\times10^{10}$ pfu/ml) in ~0.5-1 μl of PBS containing 5 mg/ml of Methyl Green was injected into the lateral ventricle of the embryonic brain using a 50 μm diameter glass micropipette connected to a 25 μl Hamilton microsyringe. After injection, 2 ml of Hank's balanced salt solution containing 0.1% glucose was added to the peritoneal cavity and the abdomen was closed.

The morning of the day on which the vaginal plug was detected was designated as E0.5.

Immunostaining of tissue sections

Embryos were fixed with 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS) for paraffin wax-embedded sectioning and frozen directly in OTC compound for frozen sectioning. Hydrolyzed paraffin wax-embedded sections were heat-treated at 120°C for 20 minutes in 10 mM sodium citrate buffer, pH 6.0. For staining with anti-BrdU antibody, sections were further treated with 1 N HCl for 15 minutes at room temperature. Frozen sections were fixed with methanol/acetone 1:1 mix at –20°C for 10 minutes and air dried. To inactivate endogenous alkaline phosphatase activity, sections were treated with 3% H_2O_2 for 15 minutes at room temperature. Immunostaining was performed according to standard protocols using 10% normal goat serum in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) as a blocking reagent, and primary and secondary antibodies diluted in 0.1% BSA/TBST, as follows. Primary antibodies: affinity purified anti-MUK rabbit antibody raised against the C-terminal part of MUK containing 276 amino acids ~1/20-1/100, omni-probe 1/300 (rabbit antibody against T7-tag, Santa Cruz), anti-class III β-tubulin ~1/50-1/200 (mouse monoclonal IgG; Chemicon), anti-MAP2 1/300 (mouse monoclonal IgG, Sigma), anti-vimentin 1/500 (mouse monoclonal IgM, Sigma), anti-active JNK/p38/MAPK (ERK) antibodies 1/1000 (rabbit, Promega), anti-α-tubulin 1/500 (mouse monoclonal IgG, Sigma), anti-GM130 1/300 (mouse monoclonal IgG, BD Transduction Lab), anti-γ-tubulin 1/300 (rabbit, Sigma) and ant-active caspase3 1/200 (rabbit, Promega). Secondary antibodies: alkaline phosphatase-conjugated anti-rabbit or mouse IgG 1/2000 (TAGO), Cy3-conjugated anti-rabbit or mouse IgG 1/2000 (Amersham), Alexa488-conjugated anti-rabbit or mouse IgG (Molecular Probe) or Cy3-conjugated anti-mouse IgM 1/2000 (Jackson Immuno Res. Lab.). DAPI (2.5 μg/ml) was included in the final wash buffer (TBST) for nuclear staining.

Protein analysis

For Western blot analysis, a pair of telencephalic vesicles from an E16 embryo were cut out in ice-cold PBS, the meninges were removed, and the vesicles were homogenized in 1 ml of SDS-PAGE sample buffer. To prepare layer-specific protein samples, thick (30 μm) sections of frozen E16 embryo head were quickly dried with a blower, and three layers containing mainly the ventricular zone, intermediate zone or cortical plate were dissected with a sharp surgical blade. Cell clumps from ~10-15 sections were lysed in 100 μl of sample buffer. These samples were appropriately diluted to give equal protein amounts and used for SDS-PAGE. Western blot analysis was performed according to standard protocols using the following antibodies: anti-MUK antibody 1/20, anti-vimentin antibody 1/500 (monoclonal IgM, Sigma), anti-MAP2 1/200 (monoclonal IgG, Sigma), anti-active JNK/p38/ERK antibodies 1/1000 (rabbit, Promega), anti-MAPK (ERK) 1/3000 (rabbit, Upstate Biochem) and anti-p38 1/300 (rabbit, Upstate Biochem). To detect JNKs, a mixture of anti-JNK1 1/1000 (mouse monoclonal, Phamingen) and anti-JNK2 1/2000 (mouse monoclonal, Santa Cruz) was used. For secondary antibodies, horseradish peroxidase-conjugated anti-rabbit or mouse Ig ~1/2000-1/5000 (Amersham) was used; the enzyme activity was detected with an ECL plus system (Amersham) and luminescence was quantified with a FUJI Las1000 plus luminescence image analyzer. The in-gel kinase assay was performed as described elsewhere (Hirai et al., 1996) using [γ^{32}P]ATP and Jun protein as a substrate; the JNK activity detected on the gel was quantified with a FUJI BAS200 image plate scanner system. β-Galactosidase protein was detected in frozen sections fixed with 0.2% glutaraldehyde at room temperature for 5 minutes using X-gal solution according to the standard protocol. The section was lightly counterstained with Hematoxylin.

Cell culture and DNA transfection

NIH3T3 cells were maintained in DMEM supplemented with 7% calf serum. For UV or serum treatment, cells were starved for 36 hours with DMEM supplemented with 0.2% fetal calf serum, and then irradiated with UVC 200 J/m^2 or fetal calf serum was added to a final concentration of 20%. Cells were lysed in SDS-PAGE sample buffer 40 minutes after UV irradiation or 5 minutes after serum addition. COS1 cells were maintained in DMEM supplemented with 10% fetal calf serum, and 3×10^6 cells were transfected with 16 μg of expression vectors for EGFP (pEGFP-c2, Clontech), T7-MUK or T7-MUK/KR by electroporation. The cells were further cultured for 24 hours and fixed with 3% PFA for 20 minutes at room temperature; immunostaining was performed as described for tissue sections. For

staining with the anti-γ-tubulin antibody, cells were fixed with methanol/acetone for 10 minutes at room temperature.

Primary cultures of neural cells were prepared from the cortical region of E16 mouse telencephalon. Cells were dispersed with 0.05% trypsin in PBS and seeded on poly-ornitin coated cover slips at a cell density of 8×10^4 cells/cm^2. After 24 hours culture in DMEM-F12 supplemented with 5% fetal calf serum, the cells were fixed and immunostained as described above.

RESULTS

MUK protein is predominantly expressed in the intermediate zone of developing mouse neocortex

We examined the expression of MUK protein in embryonic mouse telencephalon by western blot analysis using antibodies raised against the C-terminal part of the MUK protein, a region that is not conserved in other protein kinases (Hirai et al., 1996). The apparent molecular mass of the main band recognized by this antibody is 120 kDa, and the band co-migrates with recombinant MUK expressed in 293T cells (Fig. 1A). The 120 kDa band immunoprecipitated from an E16 brain extract with this antibody was recognized by a MUK antibody raised against the N-terminal part of the MUK protein (data not shown). Therefore, we conclude that the MUK protein is expressed in embryonic brain.

Immunostaining of an E16 embryo section revealed the expression of the MUK protein in neural tissues including brain, spinal cord and dorsal root ganglion (Fig. 1B). In telencephalon, MUK is highly expressed in most cells located in the intermediate zone, subventricular zone, and subplate (Fig. 1C), while it is barely detectable in the ventricular zone and cortical plate, the future neocortex. The subventricular zone and intermediate zone of the developing telencephalon consists mainly of immature neurons generated at the ventricular zone that are migrating radially towards the outermost layer of the cortical plate (Menezes and Luskin, 1994; Takahashi et al., 1996). Therefore, the predominant expression of MUK in these layers indicates the temporal expression of this protein during the radial migration of immature neurons. To confirm this, we examined MUK protein expression in developing telencephalon at different embryonic stages. MUK protein was barely detectable in the neuroepithelium surrounding the lateral ventricle at E11 (Fig. 1D), where the generation of neural cells has not yet taken place. MUK expression becomes obvious in the preplate at E13, a region composed of the earliest neurons differentiated from neuronal precursors located in the ventricular zone (Fig. 1E). The continuous generation of postmitotic neural cells and their radial migration result in the accumulation of differentiated neurons within the preplate where they start to form the cortical plate at E14. The preplate then splits into the marginal zone and subplate, which cover the pial side and ventricle side of the cortical plate, respectively (Marin-Padilla, 1978). Predominant MUK expression was found in newborn neurons in the intermediate zone located between the preplate

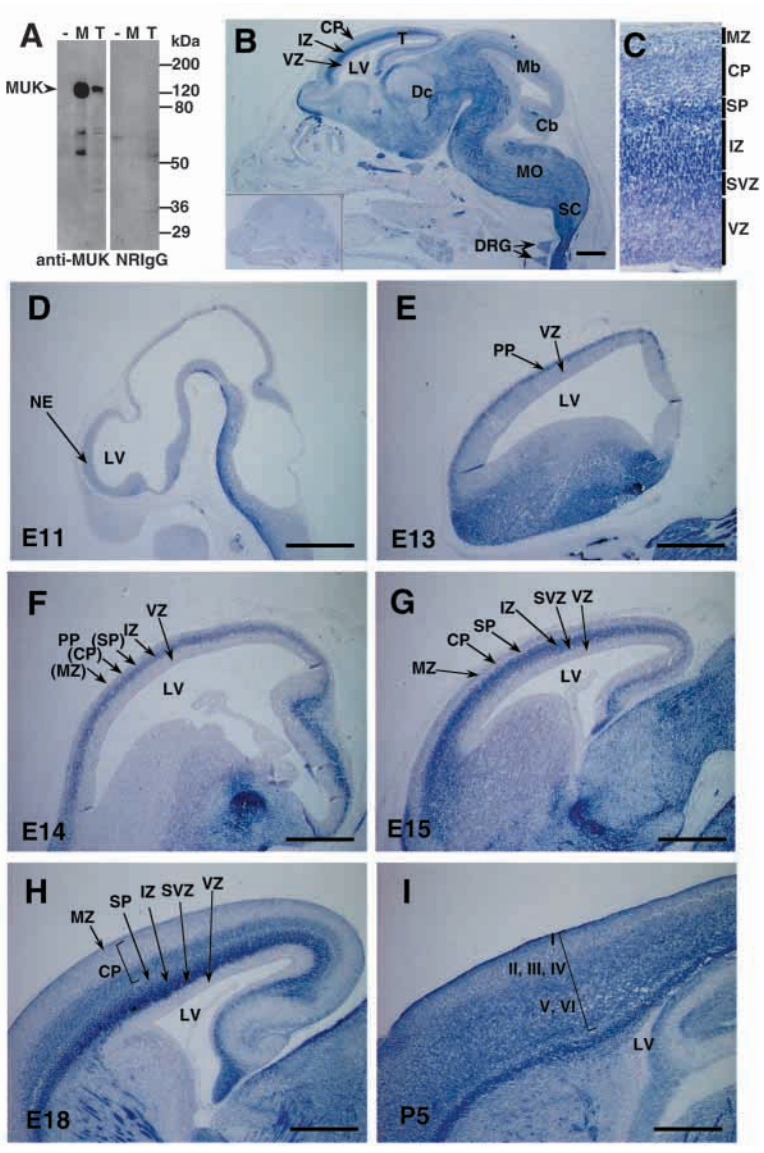

Fig. 1. The detection of MUK protein in developing mouse telencephalon. (A) Western blot analysis of proteins extracted from 293T cells (–), 293T cells overexpressing MUK (M) and E16 telencephalic vesicles (T), using affinity-purified rabbit antibody against the C-terminal 276 amino acids (left panel) or normal rabbit IgG as a control (right panel). The arrowhead indicates the position of a 120 kDa protein (MUK). (B) Sagittal sections of paraffin wax-embedded E16 embryo head were immunostained (blue) using the anti-MUK antibody, alkaline phosphatase-conjugated secondary antibody and BM purple. Positions of the telencephalon (T), diencephalon (Dc), midbrain (Mb), cerebellar primordium (Cb), medulla oblongata (MO), spinal cord (SC), dorsal root ganglia (DRG) and lateral ventricle (LV) are indicated. The insert is a control staining with normal rabbit IgG. (C) Higher magnification of the telencephalic region in B, showing predominant expression of MUK in the subplate, intermediate zone and subventricular zone. (D-I) Paraffin wax-embedded sections were prepared of the heads of E11, E13, E14 and E15 embryos and the isolated brain of an E18 embryo or 5-day-old mouse (P5) and used for the immunostaining. Distinct layers observed in the cortical region, neuroepithelium (NE), ventricular zone (VZ), pre-plate (PP), subventricular zone (SVZ), intermediate zone (IZ), subplate (SP), cortical plate (CP), marginal zone (MZ) and neocortex consisting of six presumptive layers (I-VI) are indicated. Scale bars: 500 μm.

and the ventricular zone (Fig. 1F). The MUK protein level was relatively low in the splitting preplate region and was insignificant in neural precursors of the ventricular zone. At E15 and E16, the marginal zone, cortical plate, preplate, intermediate zone, subventricular zone and ventricular zone become clearly distinguishable, and MUK protein was found to be concentrated in the subventricular zone, intermediate zone, and subplate (Fig. 1C,G). The thickness of the cortical plate and intermediate zone increases greatly at E18, and MUK expression is most prominent on the ventricle side of the intermediate zone and subventricular zone (Fig. 1H). The expression is relatively low in the outer layer of the cortical plate adjacent to the marginal zone. However, elevated levels of MUK expression were observed in the marginal zone and on the ventricle side of the cortical plate, a place where neurons differentiate at a relatively early stage (Marin-Padilla, 1978). As brain development proceeds further, MUK comes to be found in all parts of the neocortex that develop from the cortical plate and marginal zone (Fig. 1I). Taken together, these observations reveal the temporal expression of MUK in immature neurons during their radial migration, leading to stable expression in mature neurons.

JNKs are activated in cells expressing MUK

Given that MUK is an activator of the JNK pathway (Hirai et al., 1996; Fan et al., 1996), it was predicted that JNK is activated in the intermediate zone of the developing telencephalon. In fact, we were able to detect active forms of JNKs (p46 and p55) with anti-active JNK antibody when a whole telencephalon extract was used for western blot analysis (Fig. 2A, part a). To detect the active form of JNK in situ, we examined frozen sections of E16 telencephalon by immunohistochemical staining. As shown in Fig. 2B (parts a,b), the activated JNK was detected in the intermediate zone, where the expression of MUK protein was observed. A good correspondence between the localizations of MUK and active JNK in the cortex was also observed in frozen sections of an E18 embryo at (Fig. 2B, parts f,g) and other embryonic stages (data not shown). Weak signals for activated forms of p38 and ERK were also observed in the intermediate zone and cortical plate, respectively (Fig. 2B, parts c,d), while they were barely detectable by western blot analysis (Fig. 2A, parts b,c). We confirmed the preferential expression of MUK and active JNKs in the intermediate zone by western blot analysis combined with microdissection of the three cell layers composed mainly of cortical plate, intermediate zone and ventricular zone. The protein sample in the ventricular zone was characterized by an abundance of vimentin, and that in the cortical plate was characterized by an abundance of MAP2 (Fig. 2C, parts e,f). MUK protein and active JNKs were found to be highly concentrated in the intermediate zone (Fig. 2C, parts a,b), as shown by immunohistochemical staining, while the total amounts of JNK protein and total protein as estimated by Coomassie Brilliant Blue staining did not vary very much between the fractions (Fig. 2C, parts c,d). When the ratio of the amount of active- to total-JNK was calculated from the quantitative data shown in Fig. 2C (parts b,c), the value for intermediate zone JNKs was 6.02-fold (p46 JNK, s.d.=0.98, n=3) or 15.27-fold (p55 JNK, s.d.=1.75, n=3) higher than the average value for the ventricular zone and cortical plate. We further confirmed the presence of active JNK in the intermediate zone by in-gel kinase assay (Fig. 2D). Quantification of the relative JNK activity shows 5.7-fold more activity of p46 JNK and 7.7-fold more activity of p55 JNK at the intermediate zone in comparison with the average of JNK activity at the ventricular zone and cortical plate. Taken together, these observations indicate that the MUK-JNK pathway is activated temporally in migrating immature neurons.

Constitutive expression of MUK arrests the migration of immature neurons

To explore the significance of the temporally activated MUK-JNK pathway in migrating neurons, we tested the effect of MUK overexpression, which leads to the constitutive activation of JNK. Neural precursor cells were infected with an adenovirus-derived vector for MUK expression by injecting the vector into the lateral ventricle of E13 embryos. When the *lacZ* expression vector was injected as a control, most of the *lacZ*-positive cells were found in the cortical plate 3 days later, at E16, leaving some *lacZ*-positive cells in the intermediate zone (Fig. 3B). The fate of postmitotic cells that incorporated BrdU at E13, the time of adenovirus vector infection, was quite similar to that of *lacZ*-expressing cells (Fig. 3C). These results show that the adenovirus vector was carried continuously by postmitotic neurons that underwent a final mitosis just after viral infection. The virus vector carried by continuously dividing ventricular cells might be successively diluted, as the vector cannot be replicated in these cells. When a T7-tagged MUK (T7-MUK) expression vector was infected at E13, postmitotic cells expressing T7-MUK were also found in the E16 telencephalon (Fig. 3D). However, in contrast to the *lacZ*-expressing cells, all cells expressing detectable levels of T7-MUK localized in the subventricular zone and on the ventricular side of the intermediate zone (n=12). The position of these cells remained stable in E18 embryos, 5 days after vector infection (n=5) (Fig. 3G), and in embryos just before birth (data not shown). These results imply that the radial migration of T7-MUK-expressing cells was arrested at the subventricular zone and on the ventricular side of the intermediate zone. However, the overexpression of a kinase-deficient mutant of MUK (T7-MUK/KR) does not significantly impair the distribution of labeled cells; this is similar to the situation in *lacZ*-expressing cells, with biased distribution in the outermost layer of the cortical plate (n=4) (Fig. 3E). This not only supports the specificity of MUK overexpression, but also indicates that the kinase activity of T7-MUK is indispensable for the arrest of cell migration. Notably, arrest did not occur in the ventricular zone, as no cells expressing detectable amounts of T7-MUK were found in the ventricular zone at E16 or E18 (Fig. 3D,G), while both T7-MUK and T7-MUK/KR were widely expressed in most ventricular cells at E14, one-day after viral infection (Fig. 3I,J). To test if the migration arrest observed with constitutive MUK expression is specific for neurons generated at around E13, the adenovirus vector was injected at E15 and T7-MUK-expressing cells were detected at E18. As shown in Fig. 3H, again all cells expressing detectable levels of T7-MUK localized in the subventricular zone and on the ventricular side of the intermediate zone (n=3). Taken together, these observations indicate that the constitutive expression of exogenous MUK protein results in a kind of cell migration disorder characterized by the arrest of radial cell migration after the cells leave the ventricular zone.

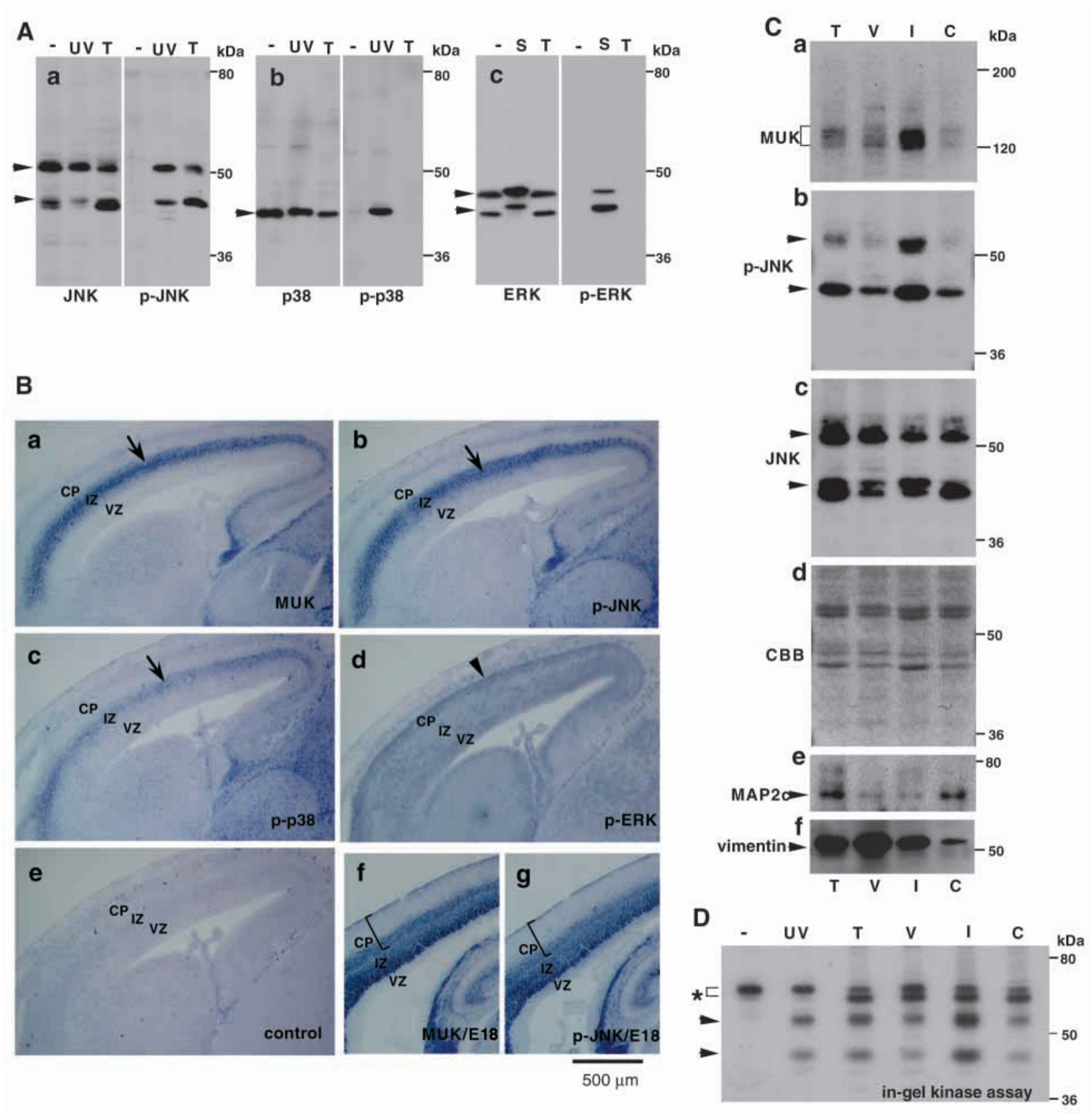

Fig. 2. The activation of JNKs at the intermediate zone. (A) Western blot analysis of proteins (10 μg) extracted from NIH3T3 cells with UV radiation (UV), serum stimulation (S) or no treatment and E16 telencephalic vesicles (T), using antibodies against JNK (a), p38 (b), ERK (c) and the active/phosphorylated form of each enzyme, p-JNK, p-p38 and p-ERK, as indicated. Arrowheads indicate protein bands corresponding to each enzyme. (B) Immunohistochemical staining of frozen sections using alkaline phosphatase-conjugated secondary antibody. Serial sagittal sections were prepared with the heads of E16 embryos (a-e) and the isolated brain of an E18 embryo (f,g) and stained with the anti-MUK antibody (a,f), anti-active MAPKs, p-JNK (b,g), p-p38 (c), p-ERK (d) or control normal rabbit IgG (e). Arrows indicate the intermediate zones stained with antibodies against MUK, p-JNK or p-p38. The arrowhead indicates the cortical plate stained with anti-p-ERK antibody. Ventricular zone (VZ), intermediate zone (IZ), and cortical plate (CP) are indicated. (C) Frozen sections were prepared from E16 telencephalon (T), and the cortical region was dissected into three layers, mainly composed of the ventricular zone (V), intermediate zone (I), and cortical plate (C). Proteins (10 μg) in each layer were separated by SDS-PAGE and analyzed by western blotting using antibodies against MUK (a), p-JNK (b), JNK (c), MAP2 (e) or vimentin (f), or stained with Coomassie Brilliant Blue (d). (D) JNK activity was detected by in-gel kinase assay using Jun protein as a substrate. The protein samples are the same as in A,C. Arrowheads indicate the positions of p46 and p55 JNKs. The asterisk indicates constitutively active protein kinases also found in unstimulated NIH3T3 cells (Hirai et al., 1996).

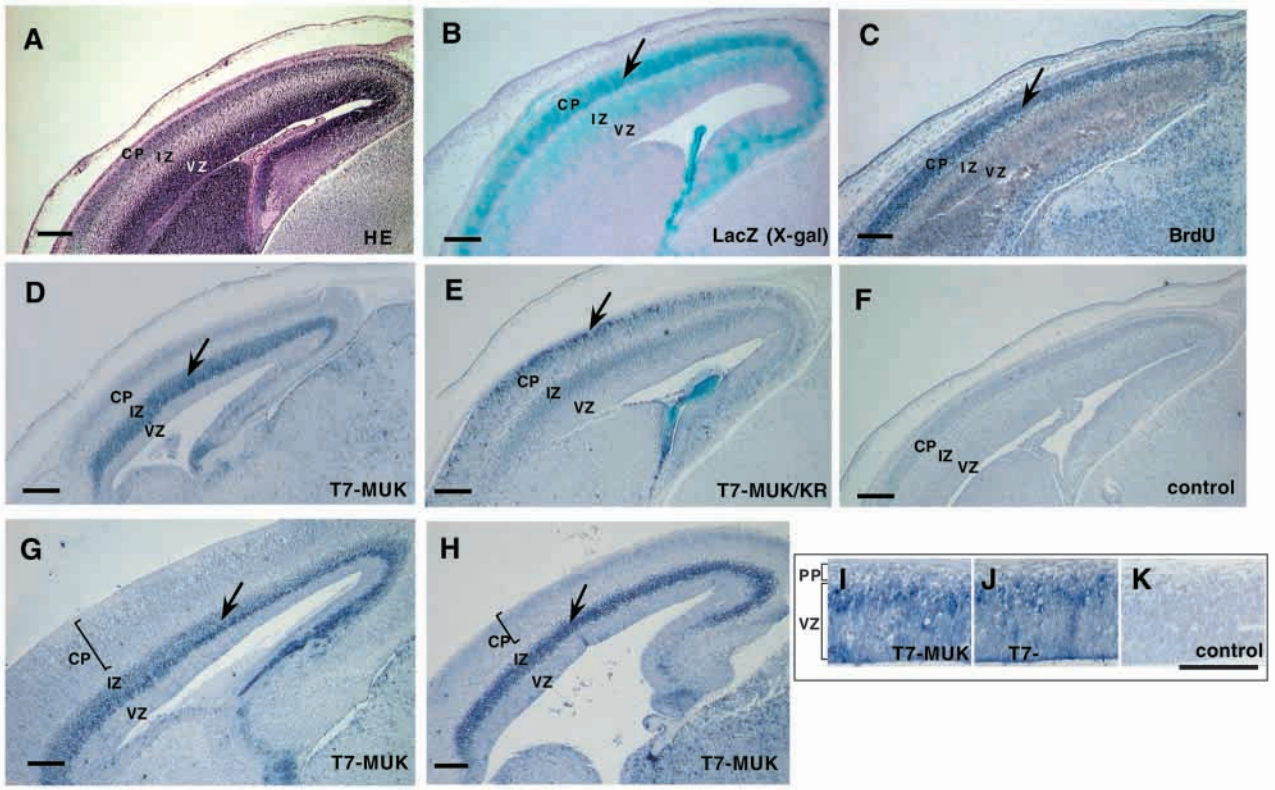

Fig. 3. Constitutive expression of MUK arrests cells at the subventricular/intermediate zone. (A) The telencephalic region in a sagittal section of a paraffin wax-embedded E16 embryo stained with Hematoxylin and Eosin, representing the ventricular zone (VZ), intermediate zone (IZ) and cortical plate (CP). (B) The adenovirus vector of *lacZ* was injected into the lateral ventricle of an E13 embryo and frozen sections of the head were prepared three days later, at E16. *lacZ* activity was detected by X-gal staining (light blue). The arrow indicates the accumulation of *lacZ*-expressing cells in the cortical plate. (C) BrdU was injected intraperitoneally into an E13-timed pregnant mouse and the embryo was fixed at E16. Paraffin wax-embedded sections of the head of the embryo were stained with anti-BrdU antibody, alkaline phosphatase-conjugated secondary antibody and BM purple. The arrow indicates the accumulation of BrdU-labeled nuclei in the cortical plate. (D) An adenovirus vector of T7-MUK was injected into the lateral ventricle of an E13 embryo and paraffin sections of the head were prepared 3 days later, at E16. Cells expressing T7-MUK were detected by immunostaining with anti-T7 antibody. The arrow indicates a cell layer accumulating T7-MUK expressing cells in the intermediate zone. (E) The adenovirus vector of T7-MUK/KR was injected and cells expressing T7-MUK/KR were detected as in D. The arrow indicates the accumulation of T7-MUK/KR expressing cells on the pial side of the cortical plate (CP). (F) A paraffin wax-embedded section prepared from a non-injected E16 embryo was immunostained as in D,E to show the background level of staining with anti-T7 antibody. (G) An adenovirus vector of T7-MUK was injected as in D, and sections were prepared at E18 for the detection of T7-MUK by immunostaining using anti-T7 antibody. The arrow indicates the remaining T7-MUK-expressing cell layer in the intermediate zone (IZ). (H) An adenovirus vector of T7-MUK was injected into the lateral ventricle of an E15 embryo and paraffin wax-embedded sections of the head were prepared three days later, at E18. The arrow indicates the T7-MUK-expressing cells accumulated in the intermediate zone (IZ). (I-K) Adenovirus vectors for T7-MUK or T7-MUK/KR were injected into the lateral ventricle of an E13 embryo. The virus-injected embryos and non-injected control embryo were fixed at E14, and paraffin wax-embedded sections were immunostained using anti-T7 antibody. Cell layers corresponding to the ventricular zone (VZ) and preplate (PP) are indicated on the left. Note the wide distribution of expression of T7-MUK and T7-MUK/KR in cells in the ventricular zone. Scale bars: 200 μm.

The migration disorder is a cell-autonomous effect of constitutive MUK expression

The radial migration of postmitotic neurons depends on radial glial cells whose cell processes, radial glial fibers, extend from the ventricular zone to the marginal zone (Rakic, 1990; Hatten, 1999). To exclude the possibility that infection with the T7-MUK expression vector affects radial glial architecture, we stained tissue sections of E16 cortex infected with adenovirus vectors using an antibody against vimentin, a marker for radial glial fibers (O'Rourke et al., 1992). As shown in Fig. 4, the overall architecture of the radial glial fibers was apparently not affected by virus vector infection. Moreover, the cortical plate of the virus infected telencephalon formed with postmitotic neurons without T7-MUK overexpression (Fig. 4A) was indistinguishable from that of uninfected control telencephalon (Fig. 4C). This indicates the normal function of radial glial fibers as a scaffold for radial cell migration in the virus infected telencephalon. Therefore, the migration disorder is most likely a cell-autonomous effect of exogenous MUK expression.

Migration-arrested cells retain features of newly generated immature neurons

Given the role of JNK in inducing apoptosis in neural cells, it is suspected that cells expressing MUK-expressing cells are undergoing apoptosis (Xu et al., 2001). To examine this possibility, we evaluated apoptotic cells 1 day or 3 days after

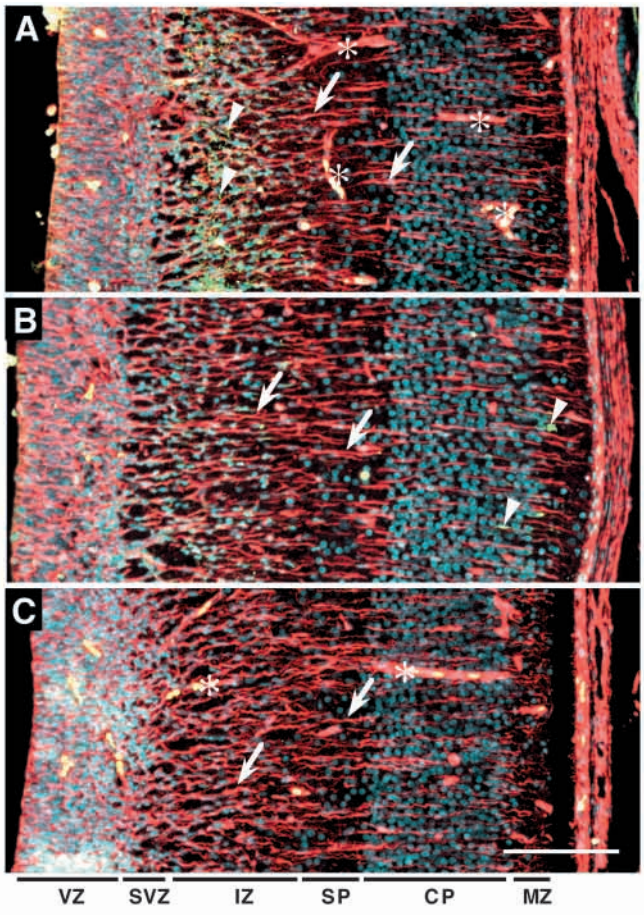

Fig. 4. Constitutive expression of MUK does not affect the radial glial fiber architecture. Adenovirus vectors were injected into the lateral ventricle of an E13 embryo and paraffin sections of the head region were prepared 3 days later, as in Fig. 3D-F. Sections were immunostained using anti-vimentin to visualize radial glial fibers (O'Rourke et al., 1992) (red). The expression of T7-MUK or T7-MUK/KR (green) and nuclear staining with DAPI (blue) are also shown. Cortical regions of the embryos infected with adenovirus vectors for T7-MUK (A) or T7-MUK/KR (B) or non-infected control embryo (C) are shown. Note that no significant changes in radial glial fiber architecture are induced by virus infection. The big yellow spots represent blood cells stained with the secondary antibodies. Arrows indicate radial glial fibers, and arrowheads indicate cells expressing T7-MUK or T7-MUK/KR. Asterisks indicate blood vessels with nonspecific staining of erythrocyte (yellow). Layer identifications are indicated at the bottom. Note the formation of the cortical plate in T7-MUK virus infected brain, as designated by a cluster of round nuclei. Scale bar: 100 μm.

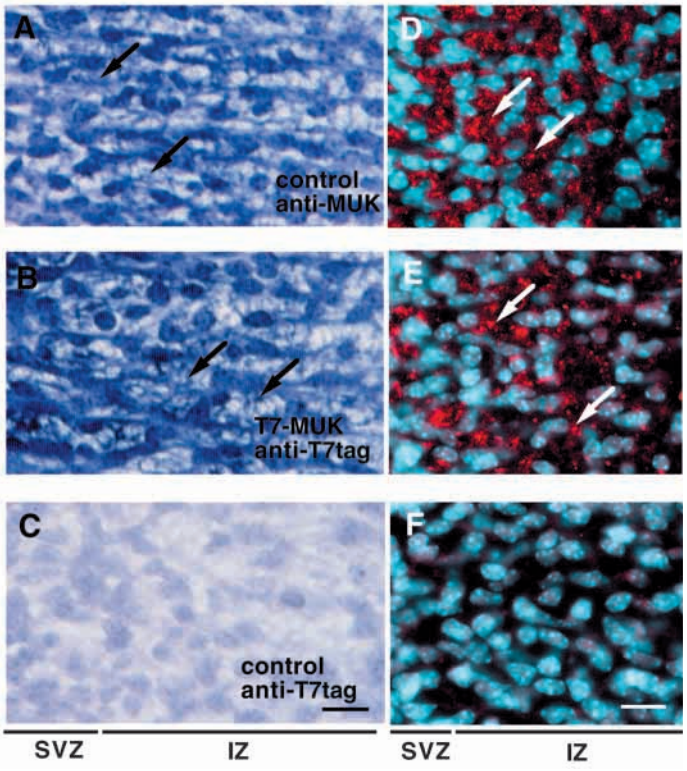

Fig. 5. Comparison of endogenous and exogenous MUK-expressing cells. (A) A section of non-infected E16 embryo telencephalon was stained with anti-MUK antibody as in Fig. 1B, to represent endogenous MUK-expressing cells. Observation of the subventricular zone (SVZ) at higher magnification reveals numerous cellular processes (arrows) as well as cell bodies stained with anti-MUK antibody. (B) A section of a T7-MUK virus-injected embryo was prepared and stained as in Fig. 3D. Observation of the subventricular zone (SVZ) at higher magnification reveals numerous cellular processes (arrows), as well as cell bodies stained with anti-T7 antibody. (C) A section of non-infected E16 embryo telencephalon was stained with anti-T7 antibody to show the background staining level. (D) A section was prepared and stained as in A, except a secondary antibody conjugated with a fluorescent dye, Cy3 (red), was used to detect endogenous MUK expression. Nuclei were labeled with DAPI (blue). Immunofluorescent microscopy reveals the localization of the MUK protein at higher resolution, associated with a dot-like structure distributing close to or distant from nuclei (arrows). (E) A section of a non-infected E16 embryo was stained as in D, using anti-T7 antibody. Arrows indicate the dot-like distribution of the T7-MUK protein. (F) A section of non-infected E16 embryo was stained as in D, using anti-T7 antibody to show the background staining level. Scale bars: 20 μm.

the injection of adenovirus vectors. One day after viral infection, E14, a significant increase in apoptotic cells was detected by anti-active-caspase 3 or nuclear condensation in the ventricular zone. However, both T7-MUK and T7-MUK/KR vectors gave similar effects. Therefore, the induction of apoptosis does not depend on the kinase activity of the expressed protein, and it is likely to be due to the toxicity of the adenovirus vector itself (data not shown). Three days after infection, E16, no apoptotic cells were found in the ventricular zone, and neither cells expressing exogenous MUK protein (T7-MUK) nor cells expressing endogenous MUK protein revealed features of apoptosis, such as cell body shrinkage or nuclear condensation or fragmentation (Fig. 5). Two days later, E18, the remaining exogenous-MUK expressing cells in the telencephalon (Fig. 3G) also were not dying.

We then compared the cell shape as well as subcellular localization of MUK proteins between cells expressing exogenous MUK and normal immature neurons expressing only endogenous MUK. Immunohistochemical staining of exogenous MUK-expressing cells revealed numerous branched processes (arrows in Fig. 5B) that are regularly found in non-infected cells in the intermediate zone stained with MUK

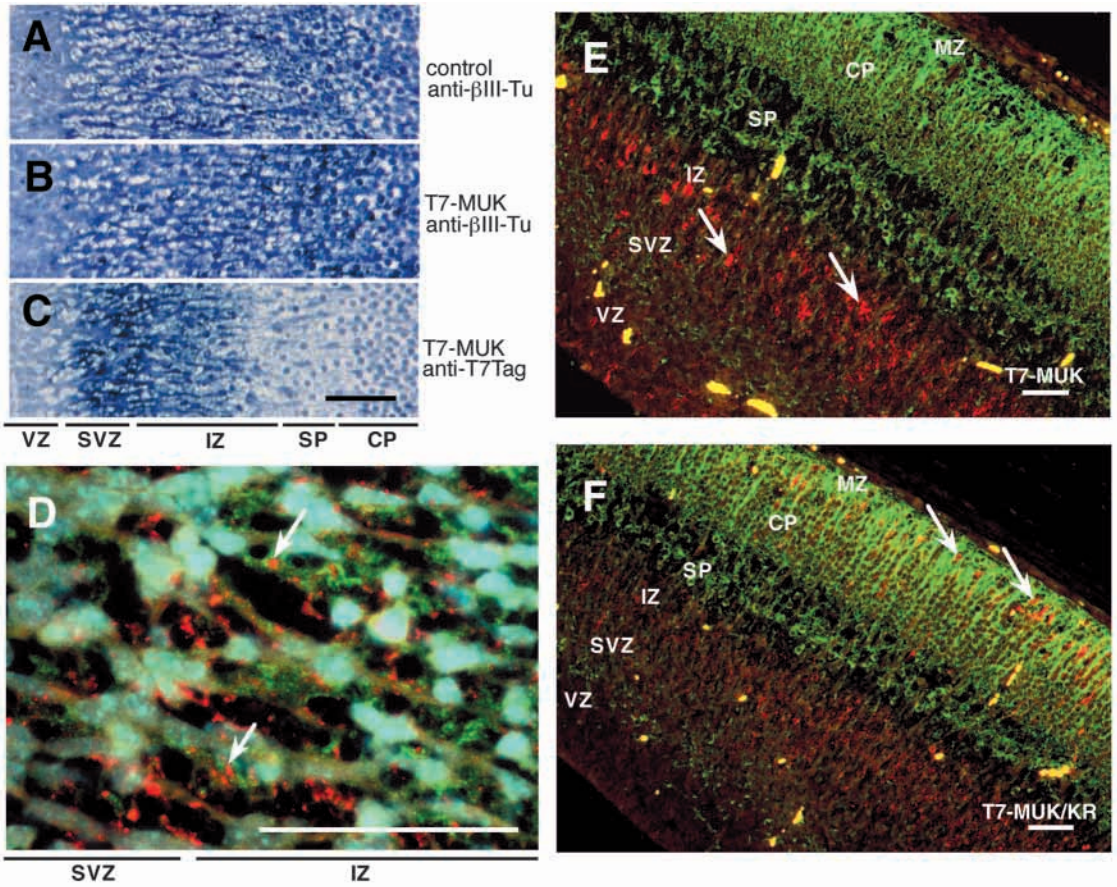

Fig. 6. Migration arrested cells show features of immature neurons. (A) A section of non-infected E16 embryonic telencephalon was stained with anti-class III β-tubulin antibody representing postmitotic immature and mature neurons (Menezes and Luskin, 1994). Note that most cells other than ventricular cells are stained. (B) A section of T7-MUK virus injected embryo was stained with anti-class III β-tubulin antibody. The staining profile is indistinguishable from that of the non-infected embryo (A). (C) A close section was stained with anti-T7 antibody. As a large region of subventricular zone (SVZ)/intermediate zone (IZ) cells are expressing distinct levels of T7-MUK, these cells may also be expressing anti-class III β-tubulin. (D) The expression of anti-class III β-tubulin in T7-MUK-expressing cells is shown by double immunostaining. Dot-like signals for T7-MUK (red) are seen on cell processes or cell bodies, representing the presence of anti-class III β-tubulin (green), as indicated by arrows. Nuclei were labeled with DAPI (blue). (E) A section of a T7-MUK virus injected embryo was double stained using anti-T7 antibody (red) and anti-MAP2 antibody (green) to show labeling of relatively mature neurons (Menezes and Luskin, 1994). Note the heavy staining of cells in the marginal zone (MZ), cortical plate (CP) and subplate (SP), and the relatively weak staining of cells in the subventricular zone (SVZ) and intermediate zone (IZ), including T7-MUK-expressing cells (arrows). (F) A section of T7-MUK/KR virus injected embryo was double stained as in E. The staining profile of MAP2 is essentially the same as in E, and in sections of non-infected embryo (data not shown). Arrows indicate T7-MUK/KR-expressing cells. Scale bars: 50 μm.

antibody (arrows in Fig. 5A). The distribution of both exogenous and endogenous MUK proteins at higher resolution detected by immunofluorescent staining exhibited a dot-like structure located along the cell body or processes (arrows in Fig. 5D,E). These results show that the exogenous MUK protein is localized correctly and does not induce significant changes in cell shape.

To characterize the differentiation stage of the exogenous MUK-expressing cells, we examined the expression of class III β-tubulin and MAP2. Class III β-tubulin is expressed in most postmitotic neurons, including immature neurons located in the subventricular and intermediate zones (Menezes and Luskin, 1994) (Fig. 6A). This expression profile is also represented in the embryonic cortex, including cells expressing exogenous MUK (Fig. 6B,C), indicating the expression of class III β-tubulin in these cells. This was confirmed by double immunofluorescent staining, showing the presence of dot-like signals for T7-MUK on the cell bodies or processes labeled with class III β-tubulin (arrows in Fig. 6D). Taken together with the observation that postmitotic cells carrying exogenous MUK protein are not found in the ventricular zone, these observations indicate that the early step of neural differentiation is not disturbed by the ectopic expression of MUK protein.

The expression of MAP2 is less prominent in immature neurons of the subventricular and intermediate zones than in mature neurons located in the marginal zone, cortical plate and subplate (Menezes and Luskin, 1994) (Fig. 6F). As shown in Fig. 6E, cells expressing exogenous MUK (red signals indicated by arrows) do not induce high levels of ectopic expression of MAP2 (green signal). In addition, the expression of GFAP, a marker protein for astroglia, was not detected in these cells as immature neurons in non-infected control telencephalon at E16 (data not shown). Consequently, the

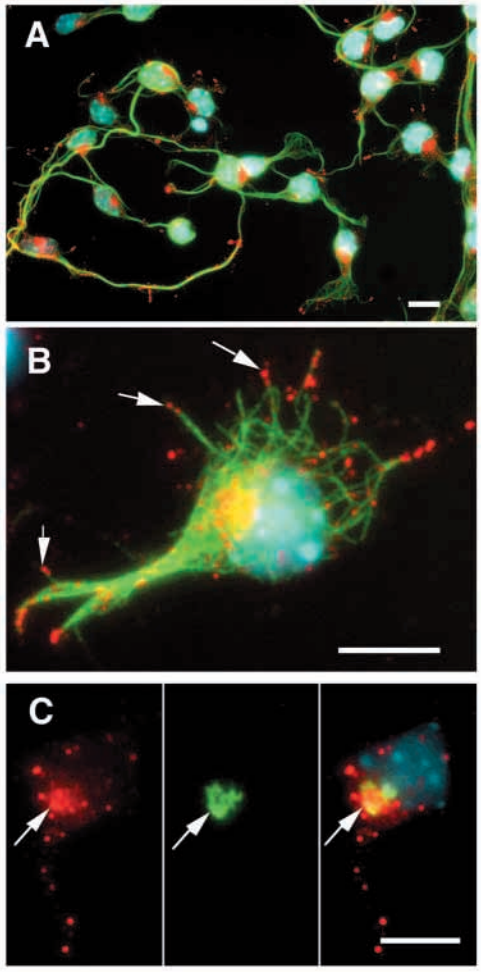

Fig. 7. MUK protein associated with microtubules and Golgi apparatus. (A) Primary culture of E16 cortical cells was stained with anti-α-tubulin antibody (green) and anti-MUK antibody (red). Nuclei were labeled with DAPI (blue). (B) Higher magnification of a cell prepared as in A, showing the association of the dotted structure containing MUK with microtubules. Many MUK-containing dotted structures are located at the tip of microtubules (arrows). (C) Cells were stained with anti-MUK antibody (left panel, red) and anti-GM130 antibody to visualize Golgi apparatus (middle panel, green). Merged view with nuclear staining with DAPI (blue) is also shown (right panel). Note the certain fraction of MUK localized on Golgi apparatus (arrows). Scale bars: 10 μm.

differentiation stage of MUK-overexpressing cells is indistinguishable from that of normal cells in the subventricular and intermediate zones that express endogenous MUK. These results indicate that migration-arrested cells expressing exogenous MUK protein correspond to cortical neurons at an early stage of differentiation rather than to aberrant cell types generated by the elevated amount of MUK protein.

MUK protein localizes on microtubules and Golgi apparatus

To understand the molecular basis of MUK function in neural cell migration, we first tested the subcellular localization of MUK using primary cultures of E16 cortical cells. To avoid continual differentiation or dedifferentiation of MUK-expressing cells in culture, dispersed cells were fixed after 24 hours of culture. As shown with tissue sections of cortex (Fig. 5D), the MUK protein associates with different sized dotted structures in primary culture cells (Fig. 7A). When merged with tubulin staining, it appeared that most of these dotted structures were located along microtubules, often at the tips of microtubules (Fig. 7B). MUK also associates with the Golgi apparatus located in the perinuclear region (Fig. 7C), as reported for NIH3T3 cells (Douziech et al., 1999).

Overexpression of MUK in COS-1 cells results in the rearrangement of microtubules

We then examined the effects of MUK overexpression on microtubule organization in COS-1 cells. Although the overexpression of MUK did not induce a breakdown of microtubules, their organization was greatly altered. The radial organization of microtubules commonly observed in COS-1 cells was rather rare in MUK overexpressing cells, and most microtubules were oriented in a random array or accumulated at the cell periphery (allows in Fig. 8A, part d). There was a significant decrease ($P<0.005$) in the presence of radial arrays of microtubules in cells expressing MUK (21.5±10.2%) compared with control GFP-expressing cells (55.8±3.9%) (Fig. 8B). However, the differences between MUK/KR-expressing cells (52.3±5.3%) and the GFP-expressing cells are not significant ($P=0.15$). Notably, centrosomes are not disrupted even in cells that had lost the radial array of microtubules (data not shown). However, microfilament organization and the formation of focal contacts were not significantly affected by MUK overexpression (data not shown).

DISCUSSION

We show that JNK activity and the expression of the neural JNK activator MUK/DLK/ZPK are induced temporally in immature neurons located in the subventricular zone and intermediate zone of developing mouse cortex. We employed a microdissection technique combined with western blot analysis to prove the results obtained by immunohistochemical staining. Importantly, cells constitutively expressing exogenous MUK/DLK/ZPK distributed continuously at the subventricular/intermediate zone, while most control cells expressing *lacZ* migrated to the cortical plate. Such typical distribution of exogenous MUK/DLK/ZPK-expressing cells most probably reflects the arrest of migration in these cells; however, it could also be caused by the total downregulation of exogenous MUK/DLK/ZPK expression in the cortical plate without migration arrest. However, the following observations strongly support the occurrence of migration arrest regardless of such downregulation in the cortical plate: (1) cells expressing T7-MUK are continuously located at the subventricular zone or intermediate zone, at least until E18, without a marked reduction in cell number (Fig. 3D,G); (2) several cells expressing a kinase-defective point mutant (T7-MUK/KR) were found on the pial side of the cortical plate, while T7-MUK expressing cell were never found in this area (Fig. 3D,E); (3) no T7-MUK-expressing cells were found in the cortical plate at E18, while the expression of endogenous MUK/DLK/ZPK became prominent (Fig. 1H, Fig. 3G).

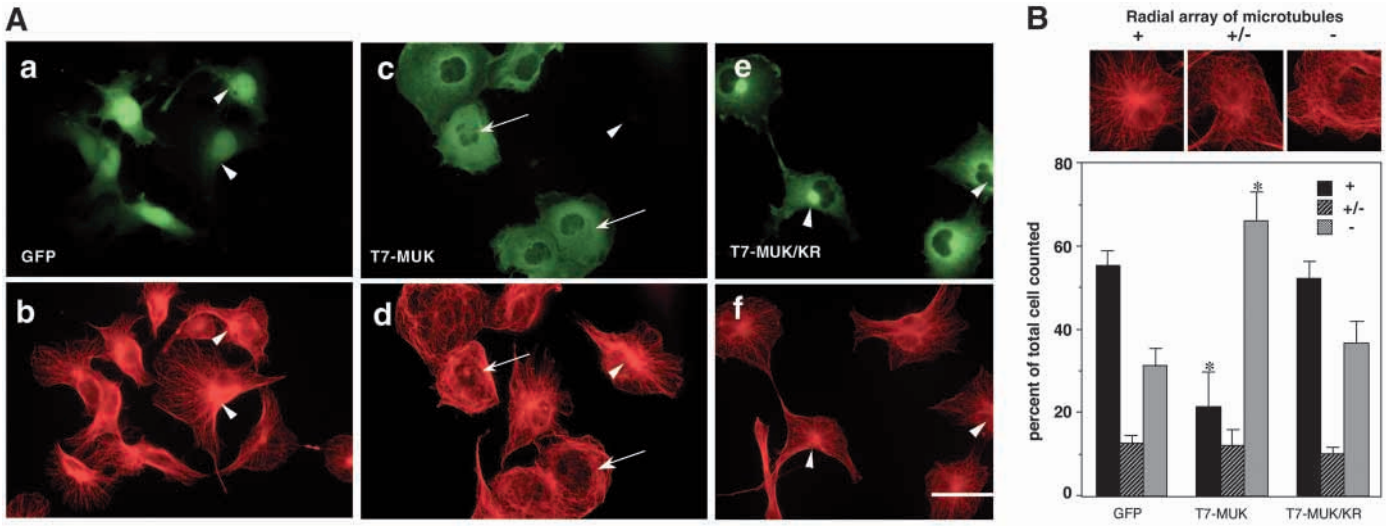

Fig. 8. Overexpression of MUK modifies microtubule organization. (A) COS-1 cells were transfected with expression vectors for GFP (a,b), T7-MUK (c,d), and T7-MUK/KR (e,f). Fixed cells were double-stained using anti-T7 antibody (c,e) (green) and anti-α-tubulin antibody (b,d,f) (red). The expression of GFP was detected by its fluorescence (a). Arrows in c and d indicate T7-MUK-expressing cells losing the radial array of microtubules. Arrowheads indicate the radial array of microtubules observed in GFP- or T7-MUK/KR-expressing cells and cells without detectable levels of T7-MUK expression. Scale bar: 50 μm. (B) The frequency of radial array appearance was analyzed statistically. Small panels shows the definition of radial array +, ± or – cells. The appearance of the radial array is expressed as percent of the GFP-, T7-MUK-, or T7-MUK/KR-positive cells counted (n>300) (*P<0.005 versus GFP or MUK/KR control). Error bars represent the standard deviation of three independent transfection experiments.

Therefore, we suggest that the MUK/DLK/ZPK-JNK pathway functions as a regulator of neural cell migration in the developing cortex, and that the activation of the MUK/DLK/ZPK-JNK pathway must be temporal in order for cell migration to be achieved.

In combination with our previous report that MUK/DLK/ZPK is a MAPKKK for the JNK pathway (Hirai et al., 1996; Fan et al., 1996), the coincidence of MUK/DLK/ZPK expression and JNK activation in the developing cortex indicates that the JNK activity is regulated by the expression level of MUK/DLK/ZPK. The preferential activation of JNK among three MAPK-related protein kinases in the E16 telencephalon is consistent with observations in COS cells overexpressing MUK/DLK/ZPK (Hirai et al., 1997; Merritt et al., 1999). The ratio of active JNK to total JNK in the intermediate zone of the E16 cortex is roughly comparable with that in NIH3T3 cells irradiated with UV, a potent activator of JNK (see Fig. 2A, part a; Fig.2C, part b; Fig. 2D), indicating the potency of JNK activity in differentiating neurons. The artificial activation of JNK during the course of sample preparation is not likely to have occurred, because samples for frozen sections and micro dissection were prepared by quick freezing in liquid nitrogen within one minute of sacrificing by cervical dislocation (see Materials and Methods). In addition, no significant changes in JNK activity were observed in E16 brain samples before or after incubation in ice-cold PBS for 30 minutes (S. H., unpublished).

How the expression of MUK/DLK/ZPK is controlled is not clear as yet. Even though the MUK/DLK/ZPK protein level is downregulated in the cortical plate of the E16 embryonic telencephalon, the mRNA level, as estimated by in situ hybridization, is still high (Nadeau et al., 1997) (S. H., unpublished). Therefore, translational or post-translational regulation in the cortical plate may be responsible for the suppression of the MUK/DLK/ZPK protein level. At a late embryonic stage, E18, the MUK/DLK/ZPK protein begins to be found on the ventricle side of the cortical plate, and it is widely expressed in all differentiated neurons in the neocortex postnatally. The MUK/DLK/ZPK-JNK pathway may therefore function not only in a temporal event in the early stages of neural differentiation, but also in general microtubule-dependent neuronal events, such as axon outgrowth and axonal transport.

Given that the MUK/DLK/ZPK-JNK pathway induces apoptosis in cultured neural cells (Xu et al., 2001), it is predicted that apoptosis is predominantly induced when cells cross the intermediate zone. However, it was difficult to detect cells meeting certain criteria for apoptosis, nuclear condensation and the expression of the active form of caspase 3, in the intermediate zone and cortical plate. Moreover, no layer-specific increase in the percentage of dying cells as detected by the in situ end-labeling method was observed in the telencephalon of normal E16 embryos (Blaschke et al., 1996). Therefore, the MUK/DLK/ZPK-JNK pathway might not induce apoptosis in the developing telencephalon.

The effect of exogenous MUK/DLK/ZPK expression is cell-autonomous, and the radial glial scaffold and radial migration of other neurons are apparently not affected. Therefore, MUK/DLK/ZPK expression may affect the interaction with radial glia and/or cell migration itself. It should also be noted that the MUK/DLK/ZPK protein associates with dotted structures that are frequently located along microtubules and that the overexpression of MUK/DLK/ZPK in COS-1 cells induces microtubule reorganization, as characterized by the disappearance of radial microtubule organization without massive depolymerization of the microtubules and disruption

of centrosomes. As a well-organized microtubule network is essential for the directed migration of cells (Gotlieb et al., 1981; DeRouvroit and Goffinet, 2001), these observations may explain, at least in part, the neuronal migration disorder observed with the constitutive expression of MUK/DLK/ZPK. This unique type of microtubule disorganization has not been commonly reported except in the case of *Lis1* overexpression in COS-7 cells (Smith et al., 2000). The *LIS1* gene is responsible for a neural cell migration disorder and type I lissencephaly in humans; the Lis1 protein associates with the dynein-dynactin complex, a microtubule motor (Smith et al., 2000; Faulkner et al., 2000; Liu et al., 2000). Therefore, the similar effects of MUK/DLK/ZPK and Lis1 overexpression on microtubule organization support the idea that the MUK/DLK/ZPK-JNK pathway regulates neural cell migration via microtubule-based event.

Genetic arguments about the function of JIPs, which are scaffold proteins for the MUK/DLK/ZPK-JNK pathway, also support this notion. Sunday driver, a *Drosophilla* homolog of JIP3/JSAP1, has recently been identified as a receptor for kinesin motors and to function in vesicle transport (Bowman et al., 2000; Verhey et al., 2001). Moreover, *Caenorhabditis elegans* JNK and JNK kinases, as well as UNC-16, a nematode homolog of JIP3, have been shown to regulate vesicle transport in neurons (Byrd et al., 2001). As vesicle transport supports cell locomotion by driving the endocytic cycle (Brestscher, 1984), JIP and JNK may be essential not only for axonal transport but also for neural cell migration. The MUK-associated dotted structures observed in the primary culture of cortical cells may correspond to vesicular cargo in secretory or endocytic pathways. Notably, mammalian JIPs bind to a Reelin receptor, ApoER2, as well as to the kinesin light chain (Stockinger et al., 2000; Verhey et al., 2001). Therefore, they may mediate the transport of vesicular cargoes containing the Reelin receptor, which is essential for the radial migration of neural cells in the cortical plate (D'Arcangelo et al., 1995; Trommsdorff et al., 1999; Senzaki et al., 1999). Even though this possibility has not been tested, our results suggest the intriguing possibility that the MUK/DLK/ZPK-JNK pathway supported by JIPs transfers a signal from Reelin to microtubules for the regulation of neural cell migration.

Although the constitutive expression of exogenous MUK/DLK/ZPK driven by the CAG promoter arrests the radial migration of neural cells, the expression of the endogenous MUK/DLK/ZPK gene does not cause such a migration disorder. This difference might be explained by the temporal features of endogenous MUK/DLK/ZPK expression, that is, the total downregulation of MUK expression on the cortical plate side of the intermediate zone. In that case, it is interesting to know whether the radially directed migration of normal immature neurons is interrupted at the intermediate zone where high levels of endogenous MUK/DLK/ZPK expression and JNK activation are observed. It has been reported that a single neural cell changes the velocity of radial migration depending on its position (O'Rourke et al., 1992; Nadarajah et al., 2001). In addition, cells in the intermediate zone often have numerous processes, rather than the single definite leading process observed in neural cells migrating in vitro or in slice cultures of neonatal ferret brain (O'Rourke et al., 1992; Rivas and Hatten, 1995). Cells with a definite leading process are found on the cortical plate side of the intermediate zone (Shoukimas and Hinds, 1978), where MUK/DLK/ZPK expression and JNK activity are significantly downregulated. Such sequential cell-shape changes of migratory neurons have been clearly shown by retrovirus vector-mediated GFP labeling of neurons in E15-E18 rat embryonic telencephalon (Noctor et al., 2001). Numerous randomly oriented cell processes are also visible by optical microscopy in MUK/DLK/ZPK expressing cells (Fig. 5A). Although the relationship between the activation of the MUK/DLK/ZPK-JNK pathway and the migration rate is not clear at present, these observations, together with the results of MUK overexpression, suggest that MUK/DLK/ZPK-JNK modulates microtubule organization and temporally disrupts the unipolar cell shape, and probably also the radial migration of immature neurons just after they leave ventricular zone (Fig. 9). This step may be required for the newly generated neurons to prepare for maturation and provide them with a chance for tangential dispersion, which is also essential for the formation of a functional neocortex (Rakic, 1990; O'Rourke et al., 1992; Parnavelas, 2000).

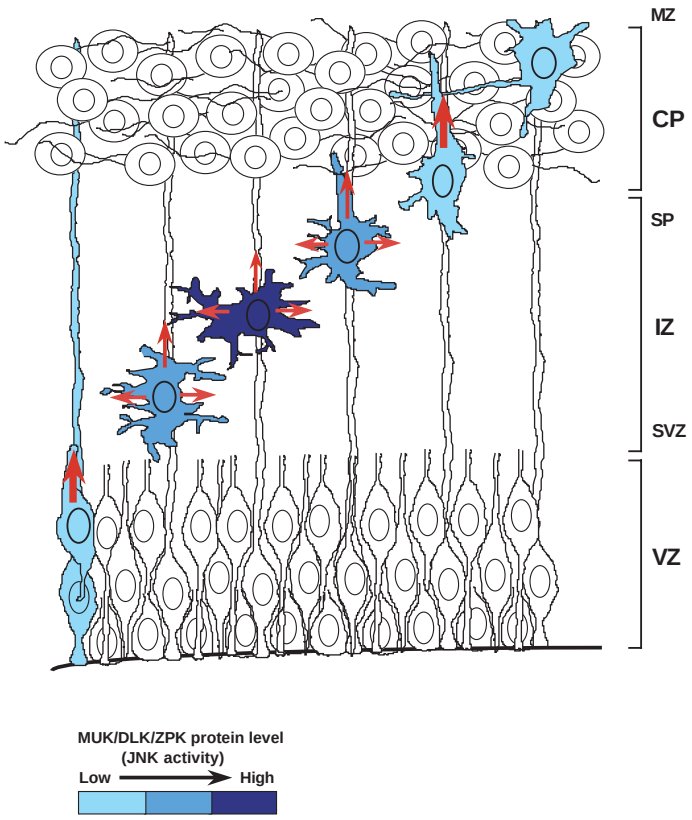

Fig. 9. Schematic drawing of radially migrating neurons in developing mouse telencephalon. Undifferentiated neuroepithelial cells located in the ventricular zone undergo asymmetrical cell division and generate postmitotic young neurons (leftmost cells). Cells leave the ventricular zone and start to differentiate and express MUK, which induces JNK activity. Simultaneously, the radial migration (vertical arrows) is retarded or pauses; this may provide a chance for tangential migration (horizontal arrows). The MUK protein level and JNK activity are reduced upon the progression of neural differentiation or by specific factors present on the pial side (upper part of this figure) of the intermediate zone. Then, a definite leading edge is formed, and radial migration is accelerated.

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