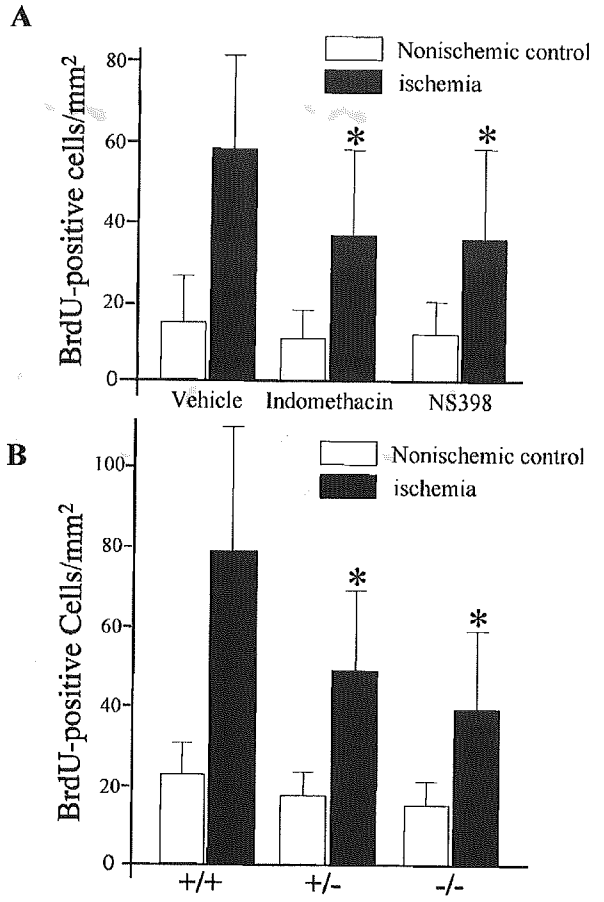

Fig. 7. Treatment with COX inhibitors or COX-2 knockout mice blunted the proliferation of progenitor cells after ischemia. Numbers of BrdU-positive cells in the SGZ are shown in nonischemic control and in ischemic mice after treatment with vehicle, indomethacin, or NS-398 **A:** Mice received BrdU (50 mg/kg, i.p.) four times every 2 hr for 6 hr starting 2 hr after the last COX inhibitor injection. They were killed 24 hr after the last BrdU administration. In contrast to the number of BrdU-positive cells in nonischemic controls (open bars), in ischemic mice (filled bars), the number of BrdU-positive cells in groups with indomethacin or NS-398 treatment in the SGZ was decreased significantly compared to vehicle treatment (n = 6 mice in each group). **B:** The number of BrdU-positive cells in controls and after ischemia in COX-2 +/+, +/−, and −/− knockout mice. In controls (open bars), the number of BrdU-positive cells in the SGZ in COX +/− (n = 6) or COX −/− (n = 4) mice were slightly less than in COX +/+ mice (n = 5), but the difference was not significant. In ischemic group (filled bars), the number of BrdU-positive cells after ischemia was significantly less in COX +/− (n = 6) or COX −/− mice (n = 5) than in COX +/+ (n = 6) mice. Data are the mean ± SD. The significance of differences was determined using ANOVA followed by Scheffe's post hoc tests. *$P < 0.05$ compared to control group.

newborn cells extended dendritic processes toward and into the molecular layer (Fig. 4). These findings indicated that, whereas cell proliferation in the dentate gyrus after an ischemic insult was transient, the resulting new neurons could be integrated into neural circuits.

COX-2 Is Expressed in Neurons and Reactive Astrocytes, But Not in Neural Progenitor Cells

In pathologic conditions, COX-2 has been shown to be induced after seizures (Yamagata et al., 1993), spreading depression (Miettinen et al., 1997), and ischemia (Nogawa et al., 1997; Iadecola et al., 2001b), and therefore may contribute to neuronal injury after brain ischemia. COX-2 mRNA and protein are upregulated immediately after either global (Ohtsuki et al., 1996; Koistinaho et al., 1999) or focal ischemia (Collaco-Moraes et al., 1996). Furthermore, prolonged COX-2 expression was observed in vulnerable neurons after global ischemia (Koistinaho et al., 1999; Matsuoka et al., 1999), and in neurons of the ischemic penumbra region after middle cerebral artery occlusion (Nogawa et al., 1997). In addition to neuronal localization, we also showed COX-2 immunoreactivity in reactive astrocytes, but not in astrocytes in control mice, in the hilus of the ischemic hippocampus, as reported previously (Degi et al., 1998; Sandhya et al., 1998; Hirst et al., 1999). Song et al. (2002) demonstrated recently that both neonatal and adult astrocytes in the hippocampus induced neurogenesis from neural stem cells. Therefore, it is also possible that COX-2 expression in astrocytes is important for the neurogenesis-inducing actions of these cells. COX-2 has been shown to be involved with cell cycle activity (Hoozemans et al., 2002; Mirjany et al., 2002). As mitotic proliferation is a critical component in adult neurogenesis, we initially estimated that neural progenitor cells themselves might express COX-2 at certain stages in their proliferation and differentiation; however, we could not detect COX-2 immunoreactivity in neural progenitors in the SGZ. Based on these findings, COX-2 in neurons and reactive astrocytes of the dentate gyrus is highly likely to be an important modulator inducing proliferation of progenitor cells in the SGZ after ischemia.

Involvement of COX-2 in Postischemic Proliferation of Neural Progenitors in the Hippocampus

In this study, enhanced proliferation of neural progenitor cells after ischemia was attenuated by treatment with COX inhibitors, and in COX-2 knockout mice. Although the suppression of enhanced neurogenesis after ischemia by administration of acetylsalicylic acid already has been reported (Kumihashi et al., 2001), acetylsalicylic acid has other COX-independent effects, such as inhibition of nuclear factor (NF) -κB, in addition to inhibiting COX (Yin et al., 1998). Therefore, our study demonstrated for the first time the involvement of COX-2 in ischemia-induced neurogenesis, although the differentiation of neural progenitor cells under COX-2 inhibition requires future clarification. The effect of the nonselective COX inhibitor indomethacin on postischemic proliferation of neural progenitor cells in the SGZ was almost the same as selective COX-2 inhibitor NS-398. This finding indicated that COX-1 as well as COX-2 might affect neural progenitor cells proliferation after ischemia. Although the role of COX-1 in ischemic brain injury has

been controversial (Iadecola et al., 2001a), it has been suggested that prostaglandin and prostacyclin produced by COX-1 play a crucial role in brain ischemia (Lin et al., 2002). Therefore, further studies are needed to determine whether COX-1 influence postischemic proliferation of neural progenitor cells.

COX-2 may affect neurogenesis in the dentate gyrus at least partly through generation of PGs, particularly PGE2, via the intermediate PGH2. It has been suggested that astrocytes could synthesize and release significant amounts of prostaglandins in vitro (Hirst et al., 1999). A recent study demonstrated that PGE2 transactivated a receptor for epidermal growth factor (EGF) and triggered mitogenic signaling (Pai et al., 2002). Multiple growth factors, including FGF-2 (Kuhn et al., 1997), EGF (Reynolds and Weiss, 1992), brain derived neurotrophic factor (BDNF) (Ahmed et al., 1995), and insulin-like growth factor (IGF) (Arsenijevic and Weiss, 1998), are known to be important regulators of proliferation and differentiation in neural stem cells. EGF receptor-like immunoreactivity was detected in proliferating cells in the dentate gyrus (Okano et al., 1996). Involvement of FGF-2 in hippocampal neurogenesis after ischemia already has been demonstrated. Involvement of FGF-2 in hippocampal neurogenesis after ischemia already has been demonstrated (Yoshimura et al., 2001). Additionally, PGE2 induced IL-6 release in astrocytes (Fiebich et al., 2001). The role of growth factors or IL-6 in neurogenesis has been already elucidated (Yoshimura et al., 2001; Vallieres et al., 2002). Interestingly, the microinjections of prostaglandin F2α (PGF2α) induced delayed VEGF expression (Skold et al., 2000). Moreover, VEGF receptor Flk-1 has been reported recently to be expressed in BrdU- and DCX-labeled immature neurons in the SGZ and SVZ (Jin et al., 2002). Based on these findings, COX-2 may influence neurogenesis in the dentate gyrus through the modulation of prostaglandin-mediated factors.

In summary, our study provides evidence that COX-2 is an important regulator in hippocampal neurogenesis in the dentate gyrus after ischemic insults. In addition, COX-2 immunoreactivity was observed in both neurons and astrocytes in the dentate gyrus, but not in neural progenitor cells in the subgranular zone. Elucidation of molecular pathways via COX-2 that underlie enhancement of neurogenesis after ischemia would be important for the therapeutic application of enhanced neurogenesis in stroke patients.

ACKNOWLEDGMENTS

This study was supported by a Grant-in-Aid for Scientific Research (B) and (C) in Japan and by Takeda Science Foundation. We thank R. Morimoto and S. Imoto for secretarial assistance.

REFERENCES

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

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

Arvidsson A, Kokaia Z, Lindvall O. 2001. *N*-methyl-D-aspartate receptor-mediated increase of neurogenesis in adult rat dentate gyrus following stroke. Eur J Neurosci 14:10–18.

Bazan NG. 2001. COX-2 as a multifunctional neuronal modulator. Nat Med 7:414–415.

Bernabeu R, Sharp FR. 2000. NMDA and AMPA/kainate glutamate receptors modulate dentate neurogenesis and CA3 synapsin-I in normal and ischemic hippocampus. J Cereb Blood Flow Metab 20:1669–1680.

Cameron HA, Gould E. 1994. Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. Neuroscience 61:203–209.

Cameron HA, McKay RD. 1999. Restoring production of hippocampal neurons in old age. Nat Neurosci 2:894–897.

Collaco-Moraes Y, Aspey B, Harrison M, de Belleroche J. 1996. Cyclo-oxygenase-2 messenger RNA induction in focal cerebral ischemia. J Cereb Blood Flow Metab 16:1366–1372.

Degi R, Bari F, Beasley TC, Thrikawala N, Thore C, Louis TM, Busija DW. 1998. Regional distribution of prostaglandin H synthase-2 and neuronal nitric oxide synthase in piglet brain. Pediatr Res 43:683–689.

Dinchuk JE, Car BD, Focht RJ, Johnston JJ, Jaffee BD, Covington MB, Contel NR, Eng VM, Collins RJ, Czerniak PM, et al. 1995. Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. Nature 378:406–409.

Fiebich BL, Schleicher S, Spleiss O, Czygan M, Hull M. 2001. Mechanisms of prostaglandin E2-induced interleukin-6 release in astrocytes: possible involvement of EP4-like receptors, p38 mitogen-activated protein kinase and protein kinase C. J Neurochem 79:950–958.

Francis F, Koulakoff A, Boucher D, Chafey P, Schaar B, Vinet MC, Friocourt G, McDonnell N, Reiner O, Kahn A, McConnell SK, Berwald-Netter Y, Denoulet P, Chelly J. 1999. Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. Neuron 23:247–256.

Gleeson JG, Lin PT, Flanagan LA, Walsh CA. 1999. Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. Neuron 23:257–271.

Gould E, Cameron HA. 1996. Regulation of neuronal birth, migration and death in the rat dentate gyrus. Dev Neurosci 18:22–35.

Gu W, Brannstrom T, Wester P. 2000. Cortical neurogenesis in adult rats after reversible photothrombotic stroke. J Cereb Blood Flow Metab 20:1166–1173.

Hayaishi O. 1991. Molecular mechanisms of sleep-wake regulation: roles of prostaglandins D2 and E2. FASEB J 5:2575–2581.

Hirst WD, Young KA, Newton R, Allport VC, Marriott DR, Wilkin GP. 1999. Expression of COX-2 by normal and reactive astrocytes in the adult rat central nervous system. Mol Cell Neurosci 13:57–68.

Hoozemans JJ, Bruckner MK, Rozemuller AJ, Veerhuis R, Eikelenboom P, Arendt T. 2002. Cyclin D1 and cyclin E are co-localized with cyclo-oxygenase 2 (COX-2) in pyramidal neurons in Alzheimer disease temporal cortex. J Neuropathol Exp Neurol 61:678–688.

Iadecola C, Niwa K, Nogawa S, Zhao X, Nagayama M, Araki E, Morham S, Ross ME. 2001b. Reduced susceptibility to ischemic brain injury and *N*-methyl-D-aspartate-mediated neurotoxicity in cyclooxygenase-2-deficient mice. Proc Natl Acad Sci USA 98:1294–1299.

Iadecola C, Sugimoto K, Niwa K, Kazama K, Ross ME. 2001a. Increased susceptibility to ischemic brain injury in cyclooxygenase-1-deficient mice. J Cereb Blood Flow Metab 21:1436–1441.

Iwai M, Sato K, Omori N, Nagano I, Manabe Y, Shoji M, Abe K. 2002. Three steps of neural stem cells development in gerbil dentate gyrus after transient ischemia. J Cereb Blood Flow Metab 22:411–419.

Jin K, Minami M, Lan JQ, Mao XO, Batteur S, Simon RP, Greenberg DA. 2001. Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat. Proc Natl Acad Sci USA 98:4710–4715.

Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA. 2002. Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. Proc Natl Acad Sci USA 99:11946–11950.

Kaneko Y, Sakakibara S, Imai T, Suzuki A, Nakamura Y, Sawamoto K, Ogawa Y, Toyama Y, Miyata T, Okano H. 2000. Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. Dev Neurosci 22:139–153.

Kaufmann WE, Andreasson KI, Isakson PC, Worley PF. 1997. Cyclooxygenases and the central nervous system. Prostaglandins 54:601–624.

Kaufmann WE, Worley PF, Pegg J, Bremer M, Isakson P. 1996. COX-2, a synaptically induced enzyme, is expressed by excitatory neurons at postsynaptic sites in rat cerebral cortex. Proc Natl Acad Sci USA 93:2317–2321.

Kitagawa K, Matsumoto M, Yang G, Mabuchi T, Yagita Y, Hori M, Yanagihara T. 1998. Cerebral ischemia after bilateral carotid artery occlusion and intraluminal suture occlusion in mice: evaluation of the patency of the posterior communicating artery. J Cereb Blood Flow Metab 18:570–579.

Koistinaho J, Koponen S, Chan PH. 1999. Expression of cyclooxygenase-2 mRNA after global ischemia is regulated by AMPA receptors and glucocorticoids. Stroke 30:1900–1906.

Kuhn HG, Winkler J, Kempermann G, Thal LJ, Gage FH. 1997. Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. J Neurosci 17:5820–5829.

Kumihashi K, Uchida K, Miyazaki H, Kobayashi J, Tsushima T, Machida T. 2001. Acetylsalicylic acid reduces ischemia-induced proliferation of dentate cells in gerbils. Neuroreport 12:915–917.

Lin H, Lin TN, Cheung WM, Nian GM, Tseng PH, Chen SF, Chen JJ, Shyue SK, Liou JY, Wu CW, Wu KK. 2002. Cyclooxygenase-1 and bicistronic cyclooxygenase-1/prostacyclin synthase gene transfer protect against ischemic cerebral infarction. Circulation 105:1962–1969.

Liu J, Solway K, Messing RO, Sharp FR. 1998. Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils. J Neurosci 18:7768–7778.

Markakis EA, Gage FH. 1999. Adult-generated neurons in the dentate gyrus send axonal projections to field CA3 and are surrounded by synaptic vesicles. J Comp Neurol 406:449–460.

Masferrer JL, Zweifel BS, Manning PT, Hauser SD, Leahy KM, Smith WG, Isakson PC, Seibert K. 1994. Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. Proc Natl Acad Sci USA 91:3228–3232.

Matsuoka Y, Okazaki M, Zhao H, Asai S, Ishikawa K, Kitamura Y. 1999. Phosphorylation of c-Jun and its localization with heme oxygenase-1 and cyclooxygenase-2 in CA1 pyramidal neurons after transient forebrain ischemia. J Cereb Blood Flow Metab 19:1247–1255.

Miettinen S, Fusco FR, Yrjanheikki J, Keinanen R, Hirvonen T, Roivainen R, Narhi M, Hokfelt T, Koistinaho J. 1997. Spreading depression and focal brain ischemia induce cyclooxygenase-2 in cortical neurons through *N*-methyl-D-aspartic acid-receptors and phospholipase A2. Proc Natl Acad Sci USA 94:6500–6505.

Mirjany M, Ho L, Pasinetti GM. 2002. Role of cyclooxygenase-2 in neuronal cell cycle activity and glutamate-mediated excitotoxicity. J Pharmacol Exp Ther 301:494–500.

Morita I, Schindler M, Regier MK, Otto JC, Hori T, DeWitt DL, Smith WL. 1995. Different intracellular locations for prostaglandin endoperoxide H synthase-1 and -2. J Biol Chem 270:10902–10908.

Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M. 2002. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. Cell 110:429–441.

Nogawa S, Zhang F, Ross ME, Iadecola C. 1997. Cyclo-oxygenase-2 gene expression in neurons contributes to ischemic brain damage. J Neurosci 17:2746–2755.

Ohtsuki T, Kitagawa K, Yamagata K, Mandai K, Mabuchi T, Matsushita K, Yanagihara T, Matsumoto M. 1996. Induction of cyclooxygenase-2 mRNA in gerbil hippocampal neurons after transient forebrain ischemia. Brain Res 736:353–356.

Okano HJ, Pfaff DW, Gibbs RB. 1996. Expression of EGFR-, p75NGFR-, and PSTAIR (cdc2)-like immunoreactivity by proliferating cells in the adult rat hippocampal formation and forebrain. Dev Neurosci 18:199–209.

Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D, Tarnawski AS. 2002. Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. Nat Med 8:289–293.

Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH. 1997. Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. J Neurosci 17:3727–3738.

Reitz DB, Li JJ, Norton MB, Reinhard EJ, Collins JT, Anderson GD, Gregory SA, Koboldt CM, Perkins WE, Seibert K, et al.. 1994. Selective cyclooxygenase inhibitors: novel 1,2-diarylcyclopentenes are potent and orally active COX-2 inhibitors. J Med Chem 37:3878–3881.

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

Sakakibara S, Imai T, Hamaguchi K, Okabe M, Aruga J, Nakajima K, Yasutomi D, Nagata T, Kurihara Y, Uesugi S, Miyata T, Ogawa M, Mikoshiba K, Okano H. 1996. Mouse-Musashi-1, a neural RNA-binding protein highly enriched in the mammalian CNS stem cell. Dev Biol 176:230–242.

Sakakibara S, Okano H. 1997. Expression of neural RNA-binding proteins in the postnatal CNS: implications of their roles in neuronal and glial cell development. J Neurosci 17:8300–8312.

Sandhya TL, Ong WY, Horrocks LA, Farooqui AA. 1998. A light and electron microscopic study of cytoplasmic phospholipase A2 and cyclooxygenase-2 in the hippocampus after kainate lesions. Brain Res 788:223–231.

Seaberg RM, van der Kooy D. 2002. Adult rodent neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors. J Neurosci 22:1784–1793.

Seri B, Garcia-Verdugo JM, McEwen BS, Alvarez-Buylla A. 2001. Astrocytes give rise to new neurons in the adult mammalian hippocampus. J Neurosci 21:7153–7160.

Shono T, Tofilon PJ, Bruner JM, Owolabi O, Lang FF. 2001. Cyclooxygenase-2 expression in human gliomas: prognostic significance and molecular correlations. Cancer Res 61:4375–4381.

Skold M, Cullheim S, Hammarberg H, Piehl F, Suneson A, Lake S, Sjogren A, Walum E, Risling M. 2000. Induction of VEGF and VEGF receptors in the spinal cord after mechanical spinal injury and prostaglandin administration. Eur J Neurosci 12:3675–3686.

Smith WL, Garavito RM, DeWitt DL. 1996. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J Biol Chem 271:33157–33160.

Song H, Stevens C, Gage F. 2002. Astroglia induce neurogenesis from adult neural stem cells. Nature 417:39–44.

Takagi Y, Nozaki K, Takahashi J, Yodoi J, Ishikawa M, Hashimoto N. 1999. Proliferation of neuronal precursor cells in the dentate gyrus is accelerated after transient forebrain ischemia in mice. Brain Res 831:283–287.

Takasawa K, Kitagawa K, Yagita Y, Sasaki T, Tanaka S, Matsushita K, Ohstuki T, Miyata T, Okano H, Hori M, Matsumoto M. 2002. Increased proliferation of neural progenitor cells but reduced survival of newborn cells in the contralateral hippocampus after focal cerebral ischemia in rats. J Cereb Blood Flow Metab 22:299–307.

Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN. 1998. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell 93:705–716.

Vallieres L, Campbell IL, Gage FH, Sawchenko PE. 2002. Reduced hippocampal neurogenesis in adult transgenic mice with chronic astrocytic production of interleukin-6. J Neurosci 22:486–492.

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH. 2002. Functional neurogenesis in the adult hippocampus. Nature 415: 1030–1034.

Yagita Y, Kitagawa K, Ohtsuki T, Takasawa K, Miyata T, Okano H, Hori M, Matsumoto M. 2001. Neurogenesis by progenitor cells in the ischemic adult rat hippocampus. Stroke 32:1890–1896.

Yamagata K, Andreasson KI, Kaufmann WE, Barnes CA, Worley PF. 1993. Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids. Neuron 11:371–386.

Yin MJ, Yamamoto Y, Gaynor RB. 1998. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. Nature 396:77–80.

Yoshimura S, Takagi Y, Harada J, Teramoto T, Thomas SS, Waeber C, Bakowska JC, Breakefield XO, Moskowitz MA. 2001. FGF-2 regulation of neurogenesis in adult hippocampus after brain injury. Proc Natl Acad Sci USA 98:5874–5879.

Zhang R, Zhang L, Zhang Z, Wang Y, Lu M, Lapointe M, Chopp M. 2001. A nitric oxide donor induces neurogenesis and reduces functional deficits after stroke in rats. Ann Neurol 50:602–611.

0023-6837/03/8304-479$03.00/0
LABORATORY INVESTIGATION

Vol. 83, No. 4, p. 479, 2003
Printed in U.S.A.

Expression of Tubulin Beta II in Neural Stem/Progenitor Cells and Radial Fibers During Human Fetal Brain Development

Yasuhiro Nakamura, Munehiko Yamamoto, Eriko Oda, Atsuyo Yamamoto, Yonehiro Kanemura, Masayuki Hara, Akira Suzuki, Mami Yamasaki, and Hideyuki Okano

Department of Pathology (YN), St. Mary's Hospital, and Department of Chemistry (MY, EO), Kurume University School of Medicine, Kurume, and Tissue Engineering Research Center (YN, MY, EO, AY, YK, MH, MY, HO), National Institute of Advanced Industrial Science and Technology, Amagasak, Department of Neuroanatomy (AS, HO), Osaka University Graduate School of Medicine, Suita, and Institute for Clinical Research (YK, MY) and Department of Neurosurgery (MY), Osaka National Hospital, Chuo-ku, Osaka, and Core Research for Evolutional Science and Technology (AS, HO), Japan Science and Technology Corporation (JST), Saitama, and Department of Physiology (HO), Keio University School of Medicine, Tokyo, Japan

SUMMARY: Recent studies revealed that the "radial glia" in fetal rodent brains are dividing neuronal precursor cells. However, in fetal primate brains, this issue remains unclear, with previous reports indicating that radial glia are a specialized form of astroglia. To investigate the relationship between radial fibers (RFs) and neural stem/progenitor cells in the fetal human brain, we generated polyclonal antibodies to human nestin protein and developed a new mAb, KNY-379, by screening for antibodies that immunostained RFs on paraffin-embedded human fetal brain specimens (12 gestational weeks). The immunostaining for KNY-379 antigen and nestin was seen over the RFs in brains at 8 gestational weeks. Furthermore, KNY-379 antigen and nestin were also detected in human neural stem/progenitor cells in neurosphere cultures. At 12 to 15 gestational weeks, the KNY-379 immunostaining of RFs remained in the periventricular zone and the deep part of the intermediate zone, but it also appeared in outgrowing axons in the cortical plate, in the superficial portion of the intermediate zone, and in apical dendrites in the molecular layer. In the later stages of fetal development (18–40 gestational weeks), this antigen remained in the outgrowing axons and dendrites, but was no longer associated with RFs. Expression cloning and immunoblot analysis demonstrated the antigen to be tubulin beta II, which would thus be a good marker for studying RFs and neural stem/progenitor cells in the early developing human brain. (*Lab Invest 2003, 83:479–489*).

In the developing mammalian brain, young postmitotic neurons leave the periventricular zone (PV) and migrate to the cortical plate (CP) along radial fibers (RFs) that extend from the cells of the periventricular neuroepithelium and terminate in branches at the pial surface of the cortex (reviewed in Bentivoglio and Mazzarello, 1999; Rakic, 1972; Walsh and Cepko, 1992). RFs are the processes of cells known as "radial cells," which have been termed differently by various researchers (His, 1889; Fujita, 1963; Noctor et al, 2001; Rakic, 1972; Ramon and Cajal, 1911). Radial cells located in the PV of the fetal monkey have been thought to be a specialized cell type belonging to the astroglial lineage, based on their expression of glial fibrillary acidic protein (GFAP) (Levitt et al, 1981) and thus have been termed "radial glia" (Bentivoglio and Mazzarello, 1999). Accordingly, RFs have been called "radial glial fibers" (Rakic, 1995).

Recent progress in neurobiology has led to a new understanding of the development and cell lineages of mammalian brain cells, specifically that neurons and glial cells derive from common progenitor cells, ie, neural stem cells (McKay, 1997). Neural stem/progenitor cells have been found in the PV in both fetal and adult human brains (Buc-Caron, 1995; Keyoung et al, 2001; Kukekov et al, 1999; Pincus et al, 1997; Roy et al, 2000). Furthermore, many lines of recent evidence suggest that some radial cells may generate neurons and that RFs are the cell processes of neural stem/progenitor cells, at least in fetal mouse brains (Hartfuss et al, 2001; Malatesta et al, 2000; Miyata et al, 2001; Noctor et al, 2001; Parnavelas and Nadarajah, 2001).

DOI: 10.1097/01.LAB.0000063930.75913.B3

Received January 22, 2003.
This study was supported by Health Sciences Research Grants for Specific Diseases "Intractable Hydrocephalus" (1999-SD-17) from the Ministry of Health and Welfare, Japan.
Address reprint requests to: Dr. Yasuhiro Nakamura, Department of Pathology, St. Mary's Hospital, 422, Tsubukuhon-machi, Kurume-shi, Japan 830-8543. E-mail: naka@st-mary-med.or.jp

Several immunohistochemical markers for RFs, including intermediate filaments, such as nestin (Hockfield and McKay, 1985; Lendahl et al, 1990) and vimentin (Kamei et al, 1998; Sarnet, 1998; Yachnis et al, 1993), have been reported. Other markers associated with RFs include antigens for the RC1 and RC2 antibodies (Edwards et al, 1990; Misson et al, 1988), brain lipid-binding protein (Feng and Heintz, 1995), bone morphogenetic protein-6 (Schluesenser and Meyermann, 1994), receptor-type protein tyrosine phosphatase-beta (Canoll et al, 1993), glutamine synthetase (Akimoto et al, 1993), and 3CB2 antigen (Prada et al, 1995). However, few studies have recently been attempted to characterize the RFs in developing human fetal brain, especially their relationship to the neural stem/progenitor cells.

To address this issue, in the present study we generated polyclonal antibodies to human nestin protein and developed a new mAb, KNY-379, which recognized RFs in situ within developing human fetal brains as well as neural progenitor cells, including neural stem cells, in human neurospheres that are enriched in these cells. Thus, at least some populations of human fetal RF are likely to be neural stem/progenitor cells. We also demonstrated by expression cloning that the antigen of KNY-379 is tubulin beta II. Thus, this study provides new tools with which to study the stem cell biology and developmental biology of the human brain.

Results

mAb KNY-379 and Polyclonal Antibody to Nestin

The antibody KNY-379 was found to belong to the IgG1, κ subclass of immunoglobulins. Both KNY-379 and polyclonal antibody to nestin reacted with paraffin-embedded human and rat tissues and with fresh-frozen sections.

Light Microscopic Immunohistochemistry

mAb KNY-379 and antibodies against vimentin (pretreated by microwaving), nestin (pretreated by incubation in a hot water bath), GFAP (pretreated by microwaving), and tubulin beta III were immunoreactive on both paraffin-embedded and fresh-frozen sections. The mAb KNY-379 demonstrated strong immunoreactivity in paraffin sections, with a longer postmortem interval equal to 12 hours (longest postmortem interval in our study).

In the cerebrum at 8 gestational weeks (GW), the distribution of the immunopositive staining with KNY-379 was similar to that seen with the antibodies to vimentin (Fig. 1, A and B) and to nestin (Fig. 1, D and E) in the PV and intermediate zone (IZ), suggesting the presence of the KNY-379 antigen along the RFs in PV and IZ. This colocalization was confirmed by double immunostaining with KNY-379 and anti-vimentin or anti-nestin antibodies (Fig. 1, C and F). Staining for tubulin beta III was negative in the PV and along the RF. The immunoreactivities with GFAP and neurofilament protein (NF) were completely negative at 8 GW.

In the cerebrum at 10 to 15 GW, positive immunostaining with KNY-379 occurred in a fibrous pattern in the PV and CP. In the IZ, the pattern of positive staining was fibrous and reticular in the deep zone of the IZ and horizontal in the superficial IZ (Fig. 2A). The pattern of immunoreactivity with anti-vimentin was similar to that of KNY-379 in the PV, but it was different in the IZ. The immunoreactivity with anti-vimentin became weak in the superficial IZ and CP (Fig. 2B). The immunoreactivity with anti-nestin was similar to that of anti-vimentin except for positive staining in the molecular layer (ML) (Fig. 2C). Tubulin beta III immunoreactivity was negative in the PV but positive in the IZ and CP. The immunoreactivities with GFAP and NF were negative between 10 and 15 weeks.

In the cerebrum at 18 to 40 GW, positive fibrous immunoreactivity with KNY-379 was confined to the CP (Fig. 3A). In the IZ, diffuse positive immunoreactivity was seen that diminished gradually. Immunoreactivity, which was probably a result of the invading ends of dendrites and axons, was strong in the ML over the cerebral convexities but somewhat weak in the sulcal depths. The positive immunoreactivity with anti-vimentin remained along the RFs as a bead-like pattern in the fading PV and in the deep zone of the IZ; but immunoreactivity was negative in the superficial IZ, CP, and ML (Fig. 3B). Nestin-positive staining was seen in the RFs of the deep IZ up to 18 GW; thereafter, the RFs were negative for nestin. The RFs were also positive with GFAP antiserum. A small number of the vimentin-positive glial progenitor cells/glioblasts were immunopositive for KNY-379. Mature astrocytes showed positive immunostaining with antibodies to vimentin and GFAP but were negative with KNY-379 and nestin on serial sections. Galactocerebroside-positive oligodendrocytes were also negative with KNY-379 and nestin on serial sections (data not shown).

Postnatally, the positive staining with KNY-379 had weakened in all layers except for the ML (not shown). The vimentin/GFAP-positive RFs disappeared after 6 months of age (not shown). The immunoreactivity with KNY-379 emerged again in some apical dendrites and/or axons of cortical neurons after 1 year of age (Fig. 4A) and in some axons in the white matter. These dendrites and axons were also positively stained with the antibody to tubulin beta III and NF (Fig. 4B). The distribution of immunoreactivity to the different antibodies is summarized in the Table.

In the cerebellum, the ML was immunopositive with KNY-379 throughout the fetal period and some apical dendrites and/or axons in the ML, the granular layer, and the white matter became positive with KNY-379 postnatally. In the brain stem and spinal cord, nerve tracts were positive for KNY-379. Positive immunoreactivity in the brain stem was present throughout the fetal, postnatal, adolescent, and adult periods. Cilia of the ependymal cells lining the ventricular system were also strongly positive with KNY-379. No positive immunoreactivity was found when the primary antibod-

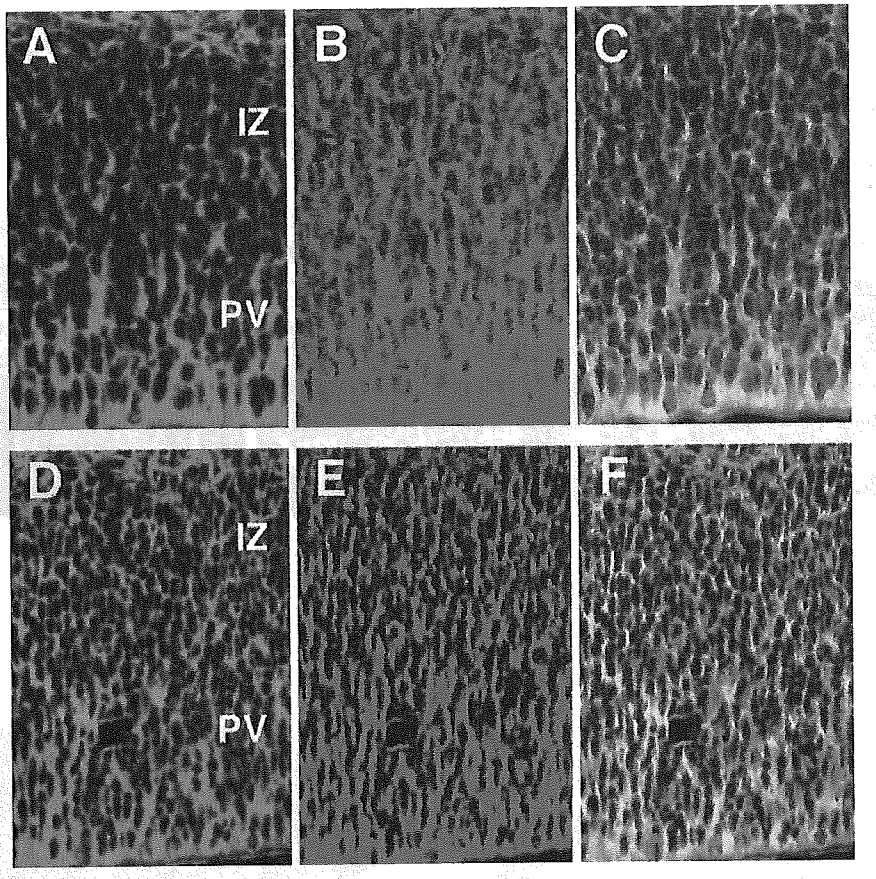

Figure 1.
Immunohistochemical distribution of tubulin beta II, vimentin, and nestin in the cerebrum at 8 gestational weeks (GW). A, Positive immunoreactivity with tubulin beta II (green) was found in the periventricular zone (PV) and intermediate zone (IZ) as a fibrous pattern. B, Positive staining with vimentin (red) was similar to that of tubulin beta II. C, Double immunostaining with tubulin beta II and vimentin suggests that both proteins were associated with radial fibers (RFs). D, Immunohistochemical distribution of tubulin beta II (green). E, Immunohistochemical distribution of nestin (red). F, Double immunostaining with tubulin beta II and nestin suggests that both proteins were associated with RFs (without counterstaining, ×100).

ies were omitted or primary antibodies preadsorbed with excess homogenate or normal sera were used.

Expressional Cloning

Three positive plaques were obtained by expression cloning, using a cDNA library from fetal brains and KNY-379, and 600 to 700 bp of each entrapped cDNA was sequenced. We searched for the cDNA sequence using the BLASTN 2.0.11 program, and a homology search was also made. All three cDNAs were identified (99–100%) as tubulin beta II. Thus, antibody KNY-379 recognizes tubulin beta II.

Immunoprecipitation and Immunoblotting

Standard Western blot showed double bands with a major band at 53 kDa and a minor band at 29 kDa in all samples (21, 27, 31, and 40 GW and adult brain). KNY-379 detected a single band at 53 kDa in immunoprecipitates in all samples (Fig. 5). The single band at 53 kDa precipitated with KNY-379 should thus correspond to tubulin beta II as reported previously (Moody et al, 1996). The bands found in standard Western blot and a single band in immunoprecipitates did not react with anti-vimentin or anti-nestin antibodies (not shown), indicating that this anti-tubulin beta II antibody did not cross-react with vimentin or nestin. A minor band at 29 kDa found in standard Western blot could be the cross-reactivity with a related protein of tubulin beta II.

Immunocytochemical Study on Human Cultured Neural Cells

The majority of cells within neurospheres stained positively with KNY-379 (tubulin beta II) as well as Musashi1 and nestin antibodies (Fig. 6). After differentiation was induced, the distribution of the immunoreactive tubulin beta II was similar to that of PSA-NCAM, indicating that it was expressed in the neural differentiated lineage (Fig. 7). A small number of tubulin beta II–positive cells also appeared to be positively immunoreactive with GFAP (Fig. 7).

Discussion

Tubulin is a major component protein of microtubules and exists as a heterodimer of two subunits designated alpha and beta. In vertebrates, the alpha and beta tubulins are encoded by small multigene families (Cleveland et al, 1980). In mammals, there are multiple

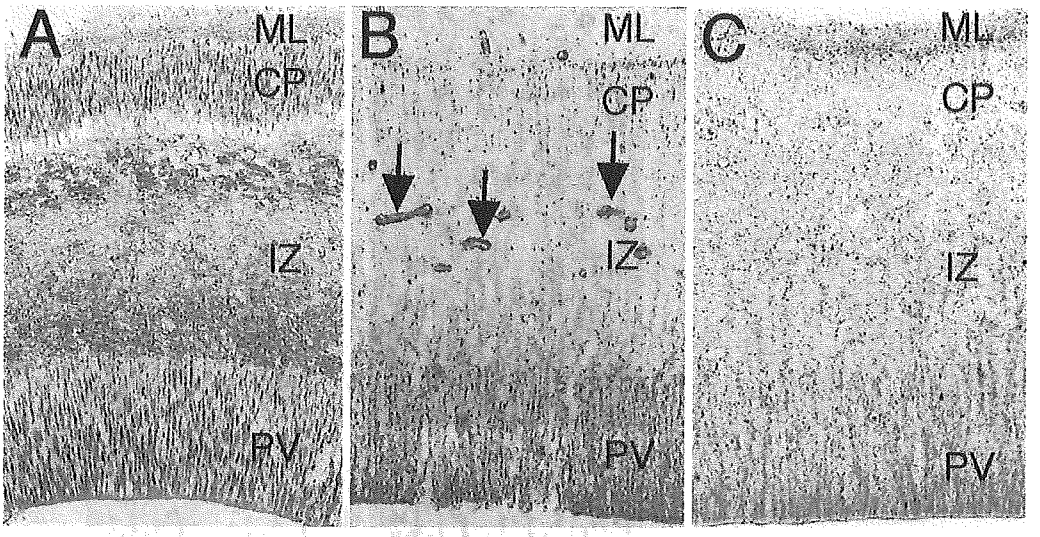

Figure 2.
Immunohistochemical distribution of tubulin beta II, vimentin, and nestin in the cerebrum at 12 GW. A, Positive immunoreactivity with tubulin beta II was fibrillary in the PV and cortical plate (CP), fibrillary/reticular in the deep zone of the IZ, and horizontal in the superficial IZ. B, Positive immunostaining for vimentin was fibrillary, but weak in the superficial IZ and CP. *Arrows* show the positive immunoreactivity in vascular walls. C, The immunoreactivity to nestin was similar to that of vimentin except for positive staining in the molecular layer (ML) (without counterstaining, ×25).

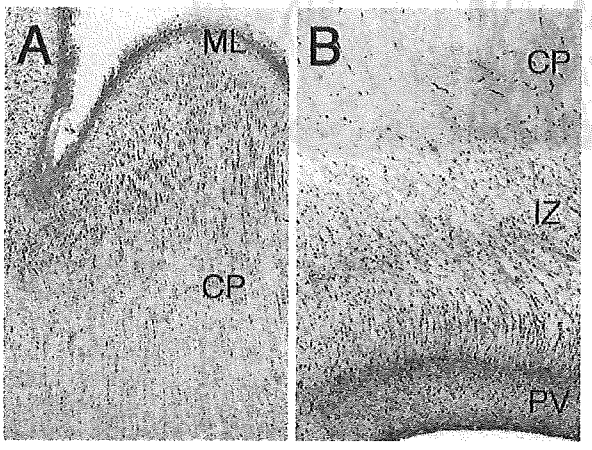

Figure 3.
Immunohistochemical distribution of tubulin beta II and vimentin in the cerebrum at 25 GW. A, positive fibrillary immunoreactivities to tubulin beta II remained only in the CP. B, Positive immunoreactivity with vimentin was found along the RF in the PV and the deep zone of the IZ but not in the superficial IZ and CP (without counterstaining: A, ×25; B, ×12).

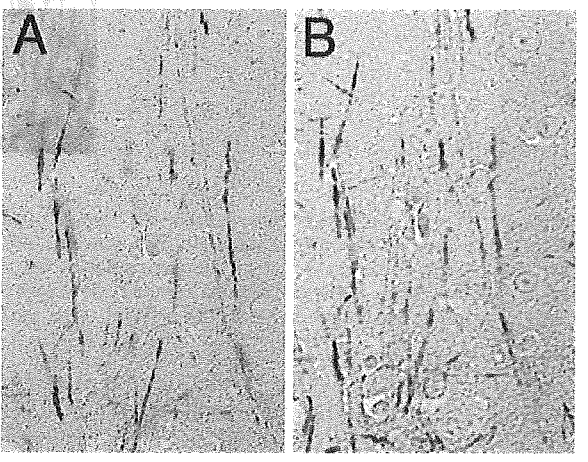

Figure 4.
Immunohistochemical distribution of tubulin beta II and neurofilament protein (NF) in the serial sections of cerebral cortex at 10 years of age. The positive immunoreactivity of tubulin beta II is seen in some apical dendrites and axons. B, Positive reactivity of NF follows the same pattern (without counterstaining, ×100).

isotypes of alpha and beta tubulin encoded by six α-tubulin and seven β-tubulin genes (Cleveland, 1987). Among the seven β-tubulins, tubulin beta I is expressed constitutively in many tissues. Beta II is the major form of tubulin beta found in neurons and is also present in many other tissues. Beta III is neuron specific, beta IVa is expressed in the nervous system and other tissues, beta IVb is expressed mainly in the testis, beta V is expressed in many tissues except for neurons, and beta VI is specific for hematopoietic tissues (Sullivan, 1988). The different β-tubulin types are highly homologous throughout most of their sequences. A major variable region exists at the carboxyl terminus (Sullivan, 1988).

The mAb KNY-379, raised against the brains of 14-day-old rat embryos, which correspond to human fetuses of 12 to 16 GW, recognizes tubulin beta II in human and rat tissues. Purified bovine brain tubulin contains tubulin beta that is comprised of 3% type I, 58% type II, 25% type III, and 13% type IV (Banerjee et al, 1988). Thus, tubulin beta II is the major form of tubulin beta in bovine brain, although it is not specific for the nervous system and is also expressed in lung, chick embryo fibroblasts, and Schwann cells (Lewis et al, 1985; Lopata and Cleveland, 1987; Robertson et al, 1992). Tubulin beta III, which is specific for neurons, has been studied extensively. The initial expression of tubulin beta III begins during or immediately after the completion of mitosis in neuroblasts (Lee et al, 1990). However, in frog embryos, tubulin beta II is the neuron-specific form and is expressed in embryonic

Table 1. Immunohistochemical Distribution of Studied Antigens

	Nes	Vim	Tub II	GFAP	Tub III	NF
8 GW						
Neural stem/progenitor cell	+	+	+	−	−	−
Radial fiber	+	+	+	−	−	−
10 to 15 GW						
Neural stem/progenitor cell	+	+	+	−	−	−
Radial fiber	+	+	+	−	−	−
Migrating neuroblast	−	−	−	−	−	−
Neuronal cells in CP	−	−	+	−	+	−
18 to 40 GW						
Radial fiber	+/−	+	−	+	−	−
Glial progenitor cells	−	+	+/−	+	−	−
Astrocyte	−	+	−	+	−	−
Oligodendrocyte	−	−	−	−	−	−
Neuronal cell	−	−	+	−	+	+
Adult brain						
Astrocyte	−	+/−	−	+	−	−
Oligodendrocyte	−	−	−	−	−	−
Neuronal cell	−	−	+	−	+	+

GW, gestational weeks; Nes, nestin; Vim, vimentin; GFAP, glial fibrillary acidic protein; Tub II, tubulin beta II; Tub III, tubulin beta III; NF, neurofilament protein; CP, cortical plate.

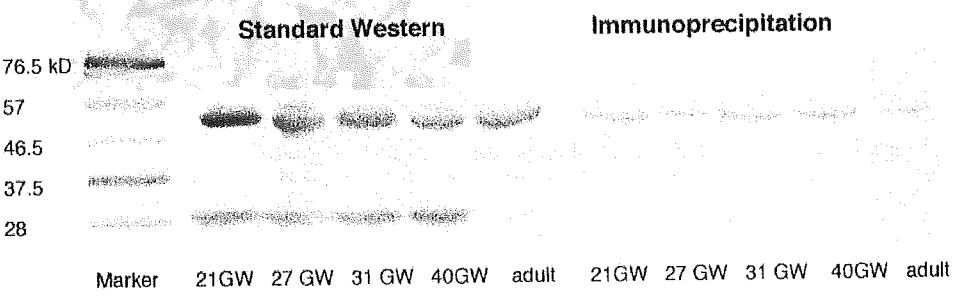

Figure 5.
Western blotting analysis of tubulin beta II expression in developing brains at 21, 27, 31, and 40 GW and in adult brain. Standard Western blot with KNY-379 showed double bands with a major band at approximately 53 kDa and a minor band at approximately 29 kDa in all brain samples. Immunoprecipitates obtained with KNY-379 showed a single band at approximately 53 kDa in all samples.

neurons but not in radial glia (Moody et al, 1996). In animal brains, tubulin beta II (Hoffman and Cleveland, 1988; Jiang and Oblinger, 1992; Paden et al, 1995) is induced significantly during development and axonal regeneration and is distributed mainly in neuronal components, but its expression in developing human brains has been little studied.

The present study clearly demonstrated that tubulin beta II, as well as vimentin and nestin, the markers for RFs, are expressed over the RFs in the human cerebrum at 8 GW. At 10 to 15 GW, the presence of tubulin beta II in the PV and the deep zone of the IZ remained the same as at 8 weeks, but the superficial zone of the IZ and CP showed a more complex pattern of positive staining, possibly reflecting tubulin beta II in outgrowing axons in addition to RFs. In the CP, although weak positive staining was seen for vimentin and nestin, staining for tubulin beta II was strongly positive as a fibrillary pattern. It was recently reported that the cortical RFs could generate neurogenesis based on a study in Pax 6 mutant mice (Heins et al, 2002). The positive signal for tubulin beta II could be attributed to dendrites and to outgrowing axons in the CP and IZ or to changes in the antigenicities of RFs themselves. The findings that the immunoreactive GFAP and NF were completely negative in developing brains during 8–15 weeks gestation may suggest that RFs and outgrowing axons are in the progenitor state without markers for mature glial and neuronal differentiation. After 18 GW, the positive fibrillary immunoreactivity of tubulin beta II remained only in the CP and had faded in the IZ. It is reported that RFs themselves remain in these structures even in the postnatal period (Yachnis et al, 1993). This study showed that vimentin and GFAP are present in RFs after 18 GW and up to 6 months after birth. RFs in this stage could be called radial glial fibers because of differentiation into glia expressing GFAP. Although vimentin was seen in astrocytes and glial progenitor cells/glioblasts, tubulin beta II was only found in some glial progenitor cells/glioblasts and not at all in mature astrocytes.

It is not clear why the positive reactivity of tubulin beta II was reduced in the cerebrum postnatally, but it may be important for the development and mainte-

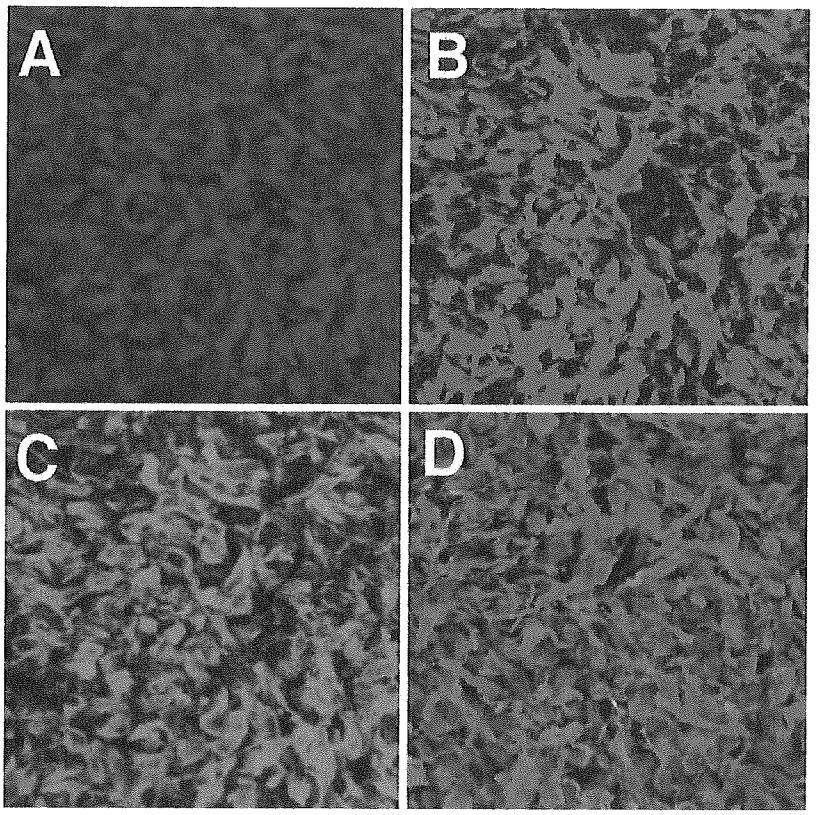

Figure 6.
Immunocytochemical distribution of Musashi1 (A, blue), nestin (B, red), and tubulin beta II (C, green) in human neurosphere cells. All three proteins were expressed in undifferentiated human neurosphere cells. D, Triple-colored immunofluorescence staining revealed the three antigens were distributed similarly in human neurosphere cells (×800).

nance of progenitor-state RFs, outgrowing axons, and dendrites in the developing brain. After cells differentiate into glia and neurons, tubulin beta II may no longer be expressed. Strong positive staining for tubulin beta II in the ML of the cerebrum and cerebellum and differences in the staining intensity in cerebral sulci suggest the important and unique roles of the ML in the developing brain. One microtubule-associated protein (MAP), MAP 5, is expressed strongly in the cerebral and cerebellar ML (Ohyu et al, 1997). MAP 2 and a phosphorylated MAP 1B are found in the developing brain and considered important for axonal outgrowth and maintenance (Honig et al, 1996; Nothias et al, 1996).

In this study, we confirmed that tubulin beta II is expressed in Musashi1 and nestin double-positive cells within neurospheres. Previously, the expression of Musashi1 and nestin was carefully studied by the infection of adenoviruses bearing the gene for green fluorescence protein in neural stem/progenitor cells, and the results showed that cells that bore both proteins were able to self renew and cogenerated neurons and glia (Keyoung et al, 2001). Neural stem cells can be selectively expanded in a serum-free defined medium containing epidermal growth factor and/or fibroblast growth factor 2, in which they give rise to a floating cell mass called a "neurosphere" (Reynolds et al, 1992; Svendsen et al, 2001). Cells within neurospheres derived from human fetal brain, which include a population with self-renewing ability and multipotency, are mostly immunopositive against anti-nestin and anti-Musashi1 antibodies. Moreover, we also observed that tubulin beta II was expressed in neuronal progenitor cells and developing neurons that express PSA-NCAM (Seki and Arai, 1993). These findings indicate that tubulin beta II is expressed in human neural stem/progenitor cells and/or neuronal lineage cells, consistent with the results from our immunohistochemical analysis.

On the other hand, in developing human brain tissue in situ after 18 GW and in differentiation-induced neuronal cells in vitro, some cells were immunopositive for both GFAP and tubulin beta II. These findings suggest that tubulin beta II is transiently expressed in glial progenitor cells (glioblasts).

The results of the present study indicate that tubulin beta II may function as a temporary constituent of progenitor-stage RFs that guide neuroblast migration and that it may be important for axonal and dendrite outgrowth thereafter, with other associated proteins such as MAP 1B and 2. We conclude that tubulin beta II is a useful marker for RFs and as such should reveal valuable information for understanding human brain development. However, the function of the tubulin beta II in neural stem/progenitor cells or RFs remains to be elucidated. Future studies using targeted disruption of the mouse tubulin beta II gene should unequivocally reveal the in vivo function of this gene product.

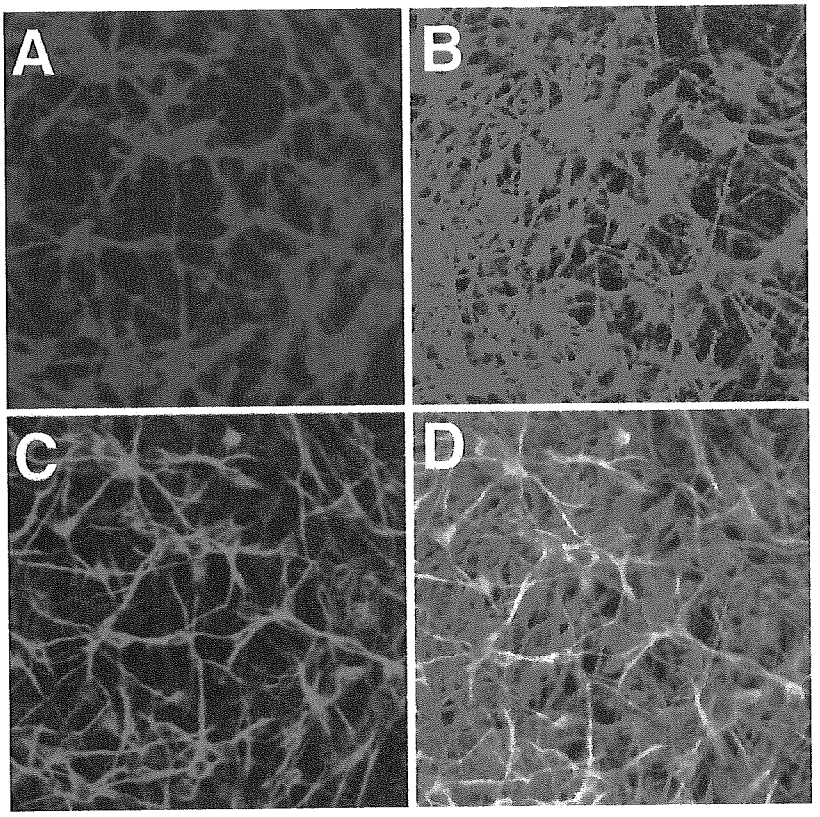

Figure 7.
Immunocytochemical distribution of PSA-NCAM (A, blue), glial fibrillary acidic protein (GFAP) (B, red), and tubulin beta II (C, green) in differentiated neural stem cells. The distribution of immunoreactivity against PSA-NCAM and tubulin beta II was quite similar. D, Triple-colored immunofluorescence staining confirmed a similar distribution of PSA-NCAM and tubulin beta II on differentiated cells, indicating neuronal differentiation. Some similarity to the distribution of GFAP was also found, suggesting that tubulin beta II might be expressed in the progenitor stage (or glioblast) of glial differentiation (×800).

Materials and Methods

Human Brain Samples

Human brain samples for immunohistochemical study were obtained from abortions and at autopsy from 32 cases ranging from 8 GW to 82 years (2 at 8 GW, 1 at 10 GW, 1 at 11 GW, 2 at 12 GW, 1 at 13 GW, 1 at 14 GW, 1 at 15 GW, 6 at 18–30 GW, 5 at 30–40 GW, 2 at 7–28 days after birth, 2 at 3 or 6 months of age, 2 at 1 year, 1 at 5 years, 1 at 10 years, and 4 at 30–82 years). All fetus samples before 20 GW were from spontaneous or electively performed abortions and were fixed in formalin. Other samples were obtained at autopsy. All cases had no abnormalities in the central nervous system. For immunohistochemistry, buffered formalin-fixed and paraffin-embedded sections were also prepared from the brains. Some samples obtained at autopsy were placed in liquid nitrogen for immunoprecipitation and Western blot preparations.

For cell culture preparations, forebrain tissue from human fetuses at 7 to 10 GW were obtained from routine legal terminations performed at the Osaka National Hospital with the approval of the ethical committee of the hospital.

Approval to use human fetal neural tissues was obtained from the ethical committees of each institution. Tissue procurement was in accordance with the Declaration of Helsinki (1964) and in agreement with the ethical guidelines of the European Network for Transplantation (NECTA) and the Japan Society of Obstetrics and Gynecology.

mAbs

For the preparation of monoclonal antibodies, homogenates were obtained from the brains of 14-day embryonal rat fetuses. Monoclonal antibodies were produced by the fusion of FO1 mouse myeloma cells with inguinal lymph node B lymphocytes from female BALB/c mice immunized with three subcutaneous injections of 200 mg of homogenate each, with the Ribi adjuvant system (Funakoshi, Tokyo) (Orlik and Altaner, 1988). Approximately 10^8 lymphocytes were obtained from the inguinal lymph nodes of 8 to 10 mice by rubbing the tissue between two frosted glass slides. The obtained lymphocytes were fused with 2 to 4×10^7 FO-1 cells by adding 50% polyethylene glycol (Sigma-Aldrich Japan, Tokyo, Japan). HAT (Sigma-Aldrich) medium selection was performed in 5 × 96-well plates with feeder cells (mice thymocytes) and growth factors (Bryclone, Dainippon-pharm, Osaka, Japan). Hybridoma culture supernatants from 480 wells were screened by incubating them with paraffin-embedded sections of developing human brain (12 GW). Approximately one fifth of supernatants were immunoreactive on the paraffin-embedded sections.

Only one clone produced antibody that reacted with RFs. It was named KNY-379 after the well number. The hybridoma cells were subcloned three times by limiting dilution and injected into the peritoneal cavity of pristane-pretreated mice. The ascites fluid was obtained approximately 1 week later. The Ig subclass was determined using a kit (Amersham Pharmacia Biotech, Sweden) according to the instructions of the manufacturer. The IgG fractions were purified on a protein G-Sepharose column. For the procedures using animals, we followed the Guide for the Care and Use of Laboratory Animals (1985).

Creation of Antibodies Against Human Nestin Protein

Antibodies against human Nestin protein were prepared as described previously (Nakanishi et al, 2001). Briefly, rabbits were immunized with a synthetic polypeptide corresponding to the C-terminal portion (1596–1610 amino acids) of the human Nestin protein (Dahlstrand et al, 1992) (GenBank accession no. X65964). To enhance its antigenicity, the peptide was covalently attached to activated keyhole limpet hemocyanin (Pierce Chemical, Rockford, Illinois) before immunization. The same Nestin peptide was immobilized on CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) for affinity purification of the peptide-specific antibodies. Immunoglobulins adsorbed to the peptide resin were eluted with 0.2 M glycine-HCl buffer (pH 2.7) and then neutralized by adding 2 M Tris-HCl buffer (pH 8.0). The specificity of the antibody was confirmed by ELISA with the peptide used for the immunization and by immunohistochemical reaction to fetal human brains (based on the characteristic staining of the RFs (Fig. 2C) and human neurosphere cells (Fig. 6B).

Light Microscopic Immunohistochemical Study

Paraffin-embedded specimens were used for immunohistochemistry according to previously reported methods (Nakamura et al, 1998) with 3-amino-9-ethylcarbazole as the chromophore. For immunohistochemical double staining, the methods are described under "Immunocytochemical Analysis" using mouse mAb KNY-379 and goat polyclonal vimentin antibody or rabbit polyclonal nestin antibody and confocal microscopy. Serial sections were used for the study of colocalization of tubulin beta II and NF or tubulin beta III or galactocerebroside. In this study, the antibody KNY-379 and antibodies against vimentin (mouse monoclonal [DAKO, Carpinteria, California] and goat polyclonal [Chemicon, Temecula, California]), nestin, GFAP (DAKO), tubulin beta III (mouse monoclonal [BABCO, Richmond, California]), NF (70 and 200 kDa; mouse monoclonal [DAKO]), and galactocerebroside (mouse monoclonal [Chemicon]) were used. In some cases, the slides were pretreated (antigen rescue) by microwaving them for 15 minutes in 10 mM citrate buffer at pH 6.0 or incubating them in a hot water bath (95° C, 40 minutes) in the same buffer. The dilutions of primary antibodies were 1:1000 (KNY-379), 1:500 (anti-tubulin beta III and nestin), and 1:200 (anti-vimentin, GFAP, galactocerebroside, and NF). For controls, the primary antibodies were omitted or primary antibodies preadsorbed with excess homogenate or normal sera were used.

Expression Cloning

A cDNA library from whole fetal human brain (male/female, 19–23 weeks, pooled) was purchased from Stratagene (La Jolla, California; catalog no. 937227). Using appropriately prepared host bacteria, XL1-Blue MRF strain, titering of lambda phages was performed according to the manufacturer's instructions. Tubes containing titered phages and host bacteria were incubated for 15 minutes at 37° C and 5 ml of LB top agarose was added. The plating culture was poured onto LB agar plates, which were then inverted and incubated overnight at 42° C. Isopropylthiogalactoside-saturated filters were placed on the surface of the agar in each plate and incubated at 37° C for 3 to 4 hours. The filters were removed, rinsed, and placed in block solution for 1 hour, then placed in KNY-379 1:1000 solution for 1 hour, rinsed, incubated with peroxidase-conjugated second antibody (anti-mouse Ig, Dako, Carpinteria, California) at a 1:1000 dilution for 1 hour, rinsed, and finally developed with 3,3'-diaminobenzidine. Positive plaques with KNY-379 antibodies were picked and subcloned three times. The cDNA was amplified by PCR with T1 and T3 primers and subcloned into pT7BlueTM T vectors (Novagen, Madison, Wisconsin), transfected into *Escherichia coli*, and sequenced.

Immunoprecipitation and Immunoblot Analysis

Tissues stored in liquid nitrogen were pulverized, washed with PBS, homogenized with lysis buffer, and immunoprecipitated using a kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions, using KNY-379. Both nonimmunoprecipitated and immunoprecipitated proteins were separated by electrophoresis on an SDS-polyacrylamide gel (5~20%), transferred to a nitrocellulose membrane, and blotted with KNY-379. Immunopositive bands were detected with an immunoperoxidase Western blotting kit (Amersham). To determine whether there was cross-reactivity with vimentin or nestin, antibodies against vimentin or nestin were also applied to the immunoblots.

Cell Culture from Human Fetal Brain Tissues

Fetal brain tissue samples were mechanically dissected in DMEM/Ham's F-12 (1:1). After dissection, the tissue samples were enzymatically digested with 0.05% trypsin/0.53 mM EDTA (Invitrogen, Carlsbad, California) for 20 minutes at 37° C. The trypsin activity was then stopped by adding soybean trypsin inhibitor (2.8 mg/ml; Beringer). Cell suspensions were grown using the neurosphere method (Reynolds et al, 1992) in defined DMEM/F-12 (1:1)-based growth medium supplemented with human recombinant epidermal growth factor (20 ng/ml; Invitrogen), human recombi-

nant fibroblast growth factor 2 (20 ng/ml; PeproTech Inc., Rocky Hill, New Jersey), human recombinant leukemia inhibitory factor (10 ng/ml; Chemicon), heparin (5 ng/ml; Sigma, St. Louis, Missouri), B27 supplement (Invitrogen), 15 mM HEPES, penicillin (100 U/ml), streptomycin (100 ng/ml), and amphotericin B (250 ng/ml). To induce differentiation, neurospheres were plated on polyornithine-coated glass coverslips and cultured in the same medium lacking the three growth factors and supplemented with 1% fetal bovine serum. The cells were cultured for 14 days before fixation for immunocytochemistry.

Immunocytochemical Analysis

Human neurospheres and coverslips with differentiated cells were fixed for 20 minutes at room temperature with 4% paraformaldehyde in PBS. After fixation, the neurospheres were dipped in 30% sucrose/PBS at room temperature for 30 minutes, embedded in OCT compound, sectioned on a cryotome at 12-μm thickness, and mounted on coverslips. The sections of neurospheres were incubated with three primary antibodies: anti-human nestin (rabbit polyclonal,1:500); anti-Musashi1 (rat monoclonal, 14H1, 1:500) (Kaneko et al, 2000), which is an evolutionally conserved neural RNA-binding protein that is selectively expressed in neural stem cells and neuronal progenitors, astroglial progenitors, and mature astrocytes (Kaneko et al, 2000; Kanemura et al, 2001; Sakakibara and Okano, 1997; Sakakibara et al, 1996); and KNY-379 in PBS/0.1% Triton-X/10% normal goat serum at 4° C overnight. The coverslips with differentiated cells were then incubated with another set of three primary antibodies: KNY-379 (1:1000), anti-PSA-NCAM (mouse IgM, 1:500; a kind gift from Dr. Seki, Department of Anatomy, Juntendo University School of Medicine) (Seki and Arai, 1993), and anti-GFAP (rabbit poly, 1:80; Sigma), under the same reaction conditions. After 3 washes, the neurosphere sections and coverslips with differentiated cells were incubated with the appropriate secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 568 goat anti-rabbit IgG, Alexa Fluor 647 goat anti-rat IgG, and Alexa Fluor 647 goat anti-mouse IgM; Molecular Probes Inc., Eugene, Oregon) at room temperature for 1 hour. The sections were then examined using a laser scanning microscope (Carl Zeiss LSM510).

Acknowledgement

We thank the staff of the Department of Pathology, St. Mary's Hospital, for their assistance.

References

Akimoto J, Itoh H, Miwa T, and Ikeda K (1993). Immunohistochemical study of glutamine synthetase expression in early glial development. Brain Res Dev Brain Res 72:9–14.

Banerjee A, Roach MC, Wall KA, Lopata MA, Cleveland DW, and Luduena RF (1988). A monoclonal antibody against the type II isotype of β-tubulin: Preparation of isotypically altered tubulin. J Biol Chem 263:3029–3034.

Bentivoglio M and Mazzarello P (1999). The history of radial glia. Brain Res Bull 49:305–315.

Buc-Caron M-H (1995). Neuroepithelial progenitor cells explanted from human fetal brain proliferate and differentiate in vitro. Neurobiol Dis 2:37–47.

Canoll PD, Barnea G, Levy JB, Sap J, Ehrlich M, Silvennoinen O, Schlessinger J, and Musacchio JM (1993). The expression of a novel receptor-type tyrosine phosphatase suggests a role in morphogenesis and plasticity of the nervous system. Brain Res Dev Brain Res 75:293–298.

Cleveland DW (1987). The multitubulin hypothesis revisited: What have we learned? J Cell Biol 104:381–383.

Cleveland DW, Lopata MA, MacDonald RJ, Cowan NJ, Rutter WJ, and Kirschner MW (1980). Number and evolutionary conservation of alpha- and beta-tubulin and cytoplasmic beta- and gamma-actin genes using specific cloned cDNA probes. Cell 20:95–105.

Dahlstrand J, Zimmerman LB, McKay RD, and Lendahl U (1992). Characterization of the human nestin gene reveals a close evolutionary relationship to neurofilaments. J Cell Sci 103:589–597.

Declaration of Helsinki (Adopted in 1964 by the 18th World Medical Assembly in Helsinki, Finland, and revised by the 29th World Medical Assembly in Tokyo in 1975). In: Andrew C. Varga, editor. The main issue in bioethics, revised edition. New York: Paulist Press, 1984.

Edwards MA, Yamamoto M, and Caviness VS Jr (1990). Organization of radial glia and related cells in the developing murine CNS: An analysis based upon a new monoclonal antibody marker. Neuroscience 36:121–144.

Feng L and Heintz N (1995). Differentiating neurons activate transcription of the brain lipid-binding protein gene in radial glia through a novel regulatory element. Development 121: 1719–1730.

Fujita S (1963). The matrix cell and cytogenesis in the developing central nervous system. J Comp Neurol 120:37–42.

Guide for the Care and Use of Laboratory Animals. NIH Publication No. 85-23 (revised 1985). Bethesda: National Institutes of Health.

Hartfuss E, Galli R, Heins N, and Gotz M (2001). Characterization of CNS precursor subtypes and radial glia. Dev Biol 229:15–30.

Heins N, Malatesta P, Cecconi F, Nakafuku M, Tucker KL, Hack MA, Chapouton P, Barde Y-A, and Gotz M (2002). Glial cells generate neurons: The role of the transcription factor Pax6. Nat Neurosci 5:308–315.

His W (1889). Die Neuroblasten und deren Entstehung im embryonalen Mark. Abh Kgl sachs Ges Wissensch math phys Kl 15:311–372.

Hockfield S and McKay RD (1985). Identification of major cell classes in the developing mammalian nervous system. J Neurosci 5:3310–3328.

Hoffman PN and Cleveland DW (1988). Neurofilament and tubulin expression recapitulates the developmental program during axonal regeneration: Induction of a specific beta-tubulin isotype. Proc Natl Acad Sci USA 85:4530–4533.

Honig LS, Herrmann K, and Shatz CJ (1996). Developmental changes revealed by immunohistochemical markers in human cerebral cortex. Cereb Cortex 6:794–806.

Jiang YQ and Oblinger MM (1992). Differential regulation of beta III and other tubulin genes during peripheral and central neuron development. J Cell Sci 103:643–651.

Kamei Y, Inagaki N, Nishizawa M, Tsutsumi O, Taketani Y, and Inagaki M (1998). Visualization of mitotic radial glial lineage cells in the developing rat brain by Cdc2 kinase-phosphorylated vimentin. Glia 23:191–199.

Kaneko Y, Sakakibara S, Imai T, Suzuki A, Nakamura Y, Sawamoto K, Ogawa Y, Toyama Y, Miyata T, and Okano H (2000). Musashi1: An evolutionally conserved marker for CNS progenitor cells including neural stem cells. Dev Neurosci 22:139-153.

Kanemura Y, Mori K, Sakakibara S, Fujikawa H, Hayashi H, Nakano A, Matsumoto T, Imai I, Ohnishi T, Fushiki F, Nakamura Y, Yamasaki M, Okano H, and Arita N (2001). Musashi1, an evolutionarily conserved neural RNA-binding protein, is a versatile marker of human glioma cells in determining their cellular origin, malignancy, and proliferative activity. Differentiation 68:141–152.

Keyoung HM, Roy NS, Benraiss A, Louissaint A Jr, Suzuki A, Hashimoto K, Rashbaum WK, Okano H, and Goldman SA (2001). High-yield selection and extraction of two promoter-defined phenotypes of neural stem cells from the fetal human brain. Nature Biotech 19:843-850.

Kukekov VG, Laywell ED, Suslov O, Davies K, Scheffler B, Thomas LB, O'Brien TF, Kusakabe M, and Steindler DA (1999). Multipotent stem/progenitor cells with similar properties arise from two neurogenic regions of adult human brain. Exp Neurol 156:333–344.

Lee MK, Tuttle JB, Rebhun LI, Cleveland DW, and Frankfurter A (1990). The expression and posttranslational modification of a neuron-specific beta-tubulin isotype during chick embryogenesis. Cell Motil Cytoskel 17:118–132.

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

Levitt P, Cooper ML, and Rakic P (1981). Coexistence of neuronal and glial precursor cells in the cerebral ventricular zone of the fetal monkey: An ultrastructural immunoperoxidase analysis. J Neurosci 1:27–39.

Lewis SA, Lee MG, and Cowan NJ (1985). Five mouse tubulin isotypes and their regulated expression during development. J Cell Biol 101:852–861.

Lopata MA and Cleveland DW (1987). In vivo microtubules are copolymers of available beta-tubulin isotypes: Localization of each of six vertebrate beta-tubulin isotypes using polyclonal antibodies elicited by synthetic peptide antigens. J Cell Biol 105:11707–11720.

Malatesta P, Hartfuss E, and Gotz M (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. Development 127:5253-5263.

McKay R (1997). Stem cells in the central nervous system. Science 276:66–71.

Misson J-P, Edwards MA, Yamamoto M, and Caviness VS Jr (1988). Identification of radial glial cells within the developing murine central nervous system: Studies based upon a new immunohistochemical marker. Brain Res Dev Brain Res 44: 95–108.

Miyata T, Kawaguchi A, Okano H, and Ogawa M (2001). Asymmetric inheritance of radial glial fibers by cortical neurons. Neuron 31:727-741.

Moody SA, Miller V, Spanos A, and Frankfurter A (1996). Development expression of a neuron-specific β-tubulin in frog (*Xenopus laevis*): A marker for growing axons during the embryonic period. J Comp Neurol 364:219–230.

Nakamura Y, Yamamoto M, and Kumamaru E (1998). A variant very low density lipoprotein receptor lacking 84 base pairs of O-linked sugar domain in the human brain myelin. Brain Res 793:47–53.

Nakanishi K, Maruyama M, Shibata T, and Morishima N (2001). Identification of a caspase-9 substrate and detection of its cleavage in programmed cell death during mouse development. J Biol Chem 276:41237–41244.

Noctor SC, Flint AC, Weissman TA, Dammerman RS, and Kriegstein AR (2001). Neurons derived from radial glial cells establish radial units in neocortex. Nature 409:714–720.

Nothias F, Fischer I, Murray M, Mirman S, and Vincent JD (1996). Expression of a phosphorylated isoform of MAP1B is maintained in adult central nervous system areas that retain capacity for structural plasticity. J Comp Neurol 368:317–334.

Ohyu J, Yamanouchi H, and Takashima S (1997). Immunohistochemical study of microtubule-associated protein 5 (MAP5) expression in the developing human brain. Brain Dev 19:541–546.

Orlik O and Altaner C (1988). Modifications of hybridoma technology which improve the yield of monoclonal antibody producing cells. J Immunol Methods 115:55–59.

Paden CM, Zhou X, Watt JA, Burton R, Pickett J, and Oblinger MM (1995). Distribution of growth-associated class I alpha-tubulin and class II beta-tubulin mRNAs in adult rat brain. J Comp Neurol 362:368–384.

Parnavelas JG and Nadarajah B (2001). Radial glial cells: Are they really glia? Neuron 31:881–884.

Pincus DW, Keyoung HM, Harrison-Restelli C, Goodman RR, Fraser RAR, Edgar M, Sakakibara S, Okano H, Nedergaard M, and Goldman SA (1997). Fibroblast growth factor-2/brain-derived neurotrophic factor-associated maturation of new neurons generated from adult human subependymal cells. Ann Neurol 43:576–585.

Prada FA, Dorado ME, Quesada A, Prada C, Schwarz U, and de la Rosa EJ (1995). Early expression of a novel radial glia antigen in the chick embryo. Glia 15:389–400.

Rakic P (1972). Mode of cell migration to the superficial layers of fetal monkey neocortex. J Comp Neurol 145:61–83.

Rakic P (1995). Radial glial cells: Scaffolding for brain construction. In: Kettenmann H and Ronsom BR, editors. Neuroglia. New York: Oxford University Press, 746–762.

Ramon Y and Cajal S (1911). In: Histologie du systeme nerveux de l'hommes et des vertebres. Paris: Maloine.

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

Robertson MD, Toews AD, Goodrum JF, and Morell P (1992). Neurofilament and tubulin mRNA expression in Schwann cells. J Neurosci Res 33:156–162.

Roy NS, Wang S, Jiang L, Kang J, Benraiss A, Harrison-Restelli C, Fraser RAR, Couldwell WT, Kawaguchi A, Okano H, Nedergaard M, and Goldman SA (2000). In vitro neurogenesis by progenitor cells isolated from the adult human hippocampus. Nat Med 6:271–277.

Sakakibara S, Imai T, Aruga J, Nakajima K, Yasutomi D, Nagata T, Kurihara Y, Uesugi S, Miyata T, Ogawa M, Mikoshiba K, and Okano H (1996). Mouse-Musashi-1, a neural RNA-binding protein highly enriched in the mammalian CNS stem cell. Dev Biol 176:230-242.

Sakakibara S and Okano H (1997). Expression of neural RNA-binding proteins in the post-natal CNS: Implication of their roles in neural and glial cell development. J Neurosci 17:8300–8312.

Sarnet HB (1998). Vimentin immunohistochemistry in human fetal brain: Methods of standard incubation versus thermal intensification achieve different objectives. Pediatr Dev Pathol 1:222–229.

Schluesenser HJ and Meyermann R (1994). Expression of BMP-6, a TGF-beta related morphogenic cytokine, in rat radial glial cells. Glia 12:161–164.

Seki T and Arai Y (1993). Distribution and possible roles of a highly polysialylated neural cell adhesion molecule (NCAM-H) in the developing and adult central nervous system. Neurosci Res 17:265–290.

Sullivan KF (1988). Structure and utilization of tubulin isotypes. Annu Rev Cell Biol 4:687–716.

Svendsen CN, Bhattacharyya A, and Tai YT (2001). Neurons from stem cells: Preventing an identity crisis. Nat Rev Neurosci 2:831–834.

Walsh C and Cepko CL (1992). Widespread dispersion of neuronal clones across functional regions of the cerebral cortex. Science 255:434–440.

Yachnis AT, Rorke LB, Lee VM-Y, and Trojanowski JQ (1993). Expression of neuronal and glial polypeptides during histogenesis of the human cerebellar cortex including observations on the dentate nucleus. J Comp Neurol 334:356–369.

Development 130, 2419-2428

doi:10.1242/dev.00468

Drosophila homeodomain protein REPO controls glial differentiation by cooperating with ETS and BTB transcription factors

Yoshihiro Yuasa[1,2], Masataka Okabe[2,3], Shingo Yoshikawa[4], Katsuhiko Tabuchi[1], Wen-Cheng Xiong[5], Yasushi Hiromi[2,3] and Hideyuki Okano[1,6,7,*]

[1]Division of Neuroanatomy (D12), Department of Neuroscience, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan
[2]Department of Developmental Genetics, National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan
[3]Department of Genetics, The Graduate University for Advanced Studies, Mishima, Shizuoka 411-8540, Japan
[4]Department of Molecular Neurobiology, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-0006, Japan
[5]Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294, USA
[6]Department of Physiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan
[7]Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation, Kawaguchi, 332-0012 Japan
*Author for correspondence (e-mail: hidokano@sc.itc.keio.ac.jp)

Accepted 10 February 2003

SUMMARY

In *Drosophila*, cell-fate determination of all neuroectoderm-derived glial cells depends on the transcription factor Glial cells missing (GCM), which serves as a binary switch between the neuronal and glial cell fates. Because the expression of GCM is restricted to the early phase of glial development, other factors must be responsible for the terminal differentiation of glial cells. Expression of three transcription factors, Reversed Polarity (REPO), Tramtrack p69 (TTK69) and PointedP1 (PNTP1), is induced by GCM in glial cells. REPO is a paired-like homeodomain protein, expressed exclusively in glial cells, and is required for the migration and differentiation of embryonic glial cells. To understand how REPO functions in glial terminal differentiation, we have analyzed the mechanism of gene regulation by REPO. We show that REPO can act as a transcriptional activator through the CAATTA motif in glial cells, and define three genes whose expression in vivo depends on REPO function. In different types of glial cells, REPO can act alone, or cooperate with either TTK69 or PNTP1 to regulate different target genes. Coordination of target gene expression by these three transcription factors may contribute to the diversity of glial cell types. In addition to promoting glial differentiation, we found that REPO is also necessary to suppress neuronal development, cooperating with TTK69. We propose that REPO plays a key role in both glial development and diversification.

Key words: *Drosophila*, Glia, Neuron, *repo*, *tramtrack*, *pointed*, *gcm*

INTRODUCTION

The two major cell types in the nervous system, neuron and glia, each comprise a large number of subtypes. In the vertebrate brain, glial cells outnumber neurons by an order of magnitude. They display a huge morphological diversity, and are involved in various functions, including supplying neurotrophic factors required for neuronal survival, the electrical insulation of axons, the formation of the blood-nerve barrier and axonal guidance. How the diversity of glial cells is generated and how these processes are linked to the cell fate choice to become a neuron or glial cell are unknown in vertebrates and invertebrates.

Glial cells in the *Drosophila* embryonic nervous system can be classified into several groups based on their location, morphology and expression of several marker genes (Ito et al., 1995). Glial cells in the central nervous system (CNS) are generated from the mesectoderm and the ventral neuroectoderm. Glial cells of mesectodermal origin are called midline glia, which unsheathe commissural axon bundles (Klämbt et al., 1991). Other CNS glia arise from glial precursors and neuroglial precursors in the ventral neuroectoderm, and consist of diverse glial types such as surface-associated glia located close to the CNS surface, cortex-associated glia that lie among the neuronal cell bodies in the cortex and neuropile-associated glia that associate with the axonal structures. Glial cells in the peripheral nervous system (PNS) are derived from either lateral neuroblasts in the ventral neuroectoderm or the dorsal epidermal anlage, and unsheathe afferent and efferent axon bundles.

In *Drosophila*, all glial cells except the midline glia require the function of the *glial cells missing* (*gcm*) gene for their cell-fate determination (Hosoya et al., 1995; Jones et al., 1995;

Vincent et al., 1996). GCM acts as a binary switch between the neuronal and glial fates; it promotes glial development, while inhibiting neuronal differentiation (Hosoya et al., 1995; Jones et al., 1995). GCM induces the expression of three transcription factors, Reversed Polarity (REPO), Tramtrack p69 (TTK69; an isoform of the *ttk* gene product) and PointedP1 (PNTP1; an isoform of the *pointed* gene product), in glial cells. Because the loss of function of any of these three factors is not as severe as the loss of GCM function, each of these factors is considered to be responsible for only a part of the GCM function. Giesen et al. (Giesen et al., 1997) proposed that glial cell differentiation is achieved by two parallel processes: the promotion of glial gene expression by PNTP1 and the suppression of neural properties by TTK69. PNTP1 is an ETS transcription factor that can activate transcription through ETS binding sites (O'Neill et al., 1994; Albagli et al., 1996; Granderath et al., 2000). It is expressed in a subset of glial cells, such as longitudinal glia and VUM glia (Klaes et al., 1994), as well as in the ventral ectoderm and tracheal cells (Mayer and Nüsslein-Volhard, 1988; Gabay et al., 1996). TTK69 is a BTB/POZ domain/zinc-finger type transcription factor that has been implicated in transcriptional repression during embryonic segmentation and eye development (Read et al., 1992; Brown and Wu, 1993; Xiong and Montell, 1993; Li et al., 1997). Its expression can be best characterized as non-neuronal; TTK69 is expressed in all cells except in the neuronal lineage, like the support cells of the sensory organ and the epidermis (Harrison and Travers, 1990; Brown et al., 1991; Read and Manley, 1992).

Although differential functions for PNTP1 and TTK69 is an attractive idea, these two factors alone cannot account for the GCM-dependent development of glial cells, because the PNTP1 and TTK69 double-mutant phenotype is different from the *gcm* mutant phenotype (Giesen et al., 1997). In addition, the pleiotropy of PNTP1 and TTK69 makes them unlikely candidates for the glial determinant. Therefore, to characterize the molecular cascades required for the differentiation of glial cells, we chose to analyze the *repo* gene, which is expressed only in GCM-positive glia (Campbell et al., 1994; Xiong et al., 1994; Halter et al., 1995). The region upstream of the *repo* transcription start site contains eleven GCM-binding consensus sequences, and GCM is necessary and sufficient to induce *repo* expression in vivo (Hosoya et al., 1995; Jones et al., 1995; Akiyama et al., 1996). Thus, *repo* is likely a direct target of GCM. REPO is a paired-like homeodomain protein that specifically binds the ATT sequence in the CAATTA motif (Halter et al., 1995). Although the expression of several glial marker genes is known to depend on *repo* activity, how REPO functions as a transcription factor in glial differentiation is as yet unknown.

We show that REPO can act as a transcriptional activator through the CAATTA motif in glial cells, and we define three genes whose expression depends on REPO function. In different types of glial cells, REPO can act alone or cooperate with either TTK69 or PNTP1 to regulate different target genes. Surprisingly, REPO also suppresses neuronal development. We propose that REPO has a cardinal function in glial identity.

MATERIALS AND METHODS

Plasmid construction, transfection and cell culture

Constructs for expression in S2 cells were made by inserting the cDNAs for *gcm*, *repo*, *repo*Δbox (with the sequence for amino acids 311-355 deleted), or the cDNA for the *pointed* P1 isoform into the pAc vector (Krasnow et al., 1989). GAL4 fusion constructs carry the GAL4 DNA binding domain (a *Hin*dIII-*Eco*RI fragment of pBD-GAL4 (Stratagene) encoding the first 147 amino acids of GAL4-) fused to a *repo* cDNA fragment, with three Myc-tags at the C terminus, in the pAc vector. The CAATTA-luc or CAGTTA-luc reporter carries two tandem copies of a 21 mer sequence, 5′-AAAGCAATTAAGCGGAACGGA-3′ or 5′-AAAGCAGTTAAGCGGAACGGA-3′ (Nelson and Laughon, 1993), upstream of the *hsp70* minimal promoter, fused to the luciferase reporter gene. The reporter gene for the GAL4 fusion proteins contains five GAL4-binding sites upstream of the *hsp70* minimal promoter fused to the luciferase gene. *loco* promoter-luciferase reporter genes AEE-luc and AES-*luc* were constructed by cloning 1.4 kb or 0.7 kb *loco* promoter fragment (extending to 6 bp upstream of the *loco-c1* translational initiation codon) into PGV-B luciferase vector (Toyo Ink). In AEE*-*luc* reporter gene, two CAATTA motifs were each changed to CAGTTA.

S2 cells cultured in a 60 mm diameter dish were transfected with 500 ng of luciferase reporter, 300 ng of effector vector and 50 ng of *Renilla* reference reporter (pACT-Rluc) using Lipofectin (GIBCO BRL) or Effectene (QIAGEN). We used the empty vector (pACT) to adjust the total amount of the effector plasmid to 300 ng, except in the AES-*luc* reporter assay, where the total was 600 ng. Luciferase activity 48 hours after transfection was normalized to values obtained with the *Renilla* reference reporter. Transfections were carried out at least three times.

Fly stocks

The following mutant alleles were used. $repo^{4e19}$ (Xiong et al., 1994), $rk2^{64}$ ($repo^{64}$) (Campbell et al., 1994), $pnt^{\Delta 88}$ (Klämbt, 1993). ttk^{1e11} mutation causes a specific loss of the TTK69 isoform (Xiong and Montell, 1993; Lai and Li, 1999) and ttk^{B330} is a strong hypomorph (Salzberg et al., 1994; Giesen et al., 1997). Homozygous mutant embryos were identified using the *TM3 [Ubx-lacZ]* balancer or by examining their phenotypes. The enhancer-trap strains pnt^{rM254} (Klämbt, 1993), ttk^{0219} (Lai et al., 1996) and $loco^{rC56}$ (Granderath et al., 1999) were used to detect the expression of *pointed*, *ttk* and *loco*, respectively. The Ftz HDS reporter (2X21F) (Nelson and Laughon, 1993) and the M84 strain (Klämbt and Goodman, 1991) were used as glial markers. Ectopic expression was achieved using GAL4 enhancer-trap insertions into *scabrous* (Kramer et al., 1995) and *engrailed* (a gift from Andrea Brand) loci. The UAS-*repo* construct was made by cloning the full-length *repo* cDNA into the pUAST vector (Brand and Perrimon, 1993). The UAS-*gcm*, UAS-*pntp1* and UAS-*ttk69* strains have been described (Hosoya et al., 1995; Klaes et al., 1994; Giesen et al., 1997).

Immunohistochemistry

Anti-REPO antiserum was obtained after immunizing rats with bacterially expressed REPO (amino acids 25-156)-GST fusion protein. Immunohistochemistry was performed as described by Patel (Patel, 1994) with minor modifications. The following primary antibodies were used: mouse anti-BP102 [obtained from Developmental Studies Hybridoma Bank (DSHB)] at 1:4, mouse anti-ELAV at 1:4 (obtained from DSHB), anti-REPO rat antiserum at 1:1000, rat anti-TTK69 at 1:200 (gift from A. Travers) and rabbit anti-β-Galactosidase polyclonal antibody (Cappel) at 1:1000. The following secondary antibodies were used: HRP-conjugated goat anti-mouse, anti-rat, or anti-rabbit IgG (Jackson ImmunoResearch) at 1:1000, and FITC- conjugated donkey anti-rat, Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch) at 1:800. Immunofluorescence was visualized with a FLUOVIEW laser-scanning microscope (Olympus). Embryos were staged according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997).

In situ hybridization

Whole-mount in situ hybridization was conducted using digoxigenin-

labeled RNA probes, essentially as described in Lehmann and Tautz (Lehmann and Tautz, 1994). Full-length *loco-cl* cDNA was obtained by RT-PCR amplification and was used as a template.

RESULTS

REPO is a transcriptional activator

Based on the observation that a *lacZ* reporter gene with a CAATTA homeodomain consensus binding site (*ftz* HDS reporter) was specifically expressed in glial cells, Nelson and Laughon (Nelson and Laughon, 1993) had predicted the presence of a homeodomain transcriptional activator in glial cells. The homeodomain protein REPO is a good candidate for such an activator, because it binds to the CAATTA motif in vitro (Halter et al., 1995), and its expression is confined to glial cells (Campbell et al., 1994; Xiong et al., 1994; Halter et al., 1995). To test whether REPO indeed functions as a transcriptional activator, we tested the transcriptional regulatory activity of REPO in culture cells.

We constructed a luciferase reporter gene with two REPO-binding sites (CAATTA-luc) and tested its transcriptional activity in the *Drosophila* S2 cell line. Co-transfection with a REPO-expressing plasmid (pACT-repo) caused a sevenfold increase in luciferase activity compared with co-transfection with the vector alone (pACT) (Fig. 1A). REPO lacking most of its homeodomain, but retaining a putative nuclear localization signal located from amino acid 1 to 4 of the homeobox (pACT-repoΔbox), was unable to activate transcription of the reporter gene (Fig. 1A), despite being localized to the nucleus (data not shown). Furthermore, transcriptional activation by REPO was dependent on the presence of the CAATTA motif in the reporter gene; a single base substitution in this motif resulted in a complete loss of REPO-dependent transcription (Fig. 1A). We conclude that REPO is a transcriptional activator that can act through the CAATTA motif.

To identify the transcriptional activation domain of REPO, fusion proteins of various segments of REPO with the DNA-binding domain of GAL4 were expressed in S2 cells, and their transcriptional activation activity was assayed by measuring the enzymatic activities of the UAS-luciferase reporter gene. Fusion of the full-length REPO to the GAL4 DNA-binding domain caused a 2.4-fold activation of the reporter gene compared with the GAL4 DNA-binding domain alone. We identified two non-overlapping segments of REPO (amino acids 1-219, 452-612) that had significant levels of transactivation activity. Deletion of either segment retained the transcriptional activation activity present in the full-length fusion, suggesting that REPO may contain regions that inhibit the function of its activation domains. Indeed, deleting the N-terminal 124 amino acids caused an increase in activation that was more than 20-fold, indicating that a strong inhibitory domain is present in the N terminus. The presence of multiple functional domains suggests that REPO may employ different mechanisms of transcriptional regulation depending on the cellular context.

REPO functions as a transcriptional activator in glial cells

To test whether REPO activates transcription through CAATTA sites in vivo, we examined the expression pattern of

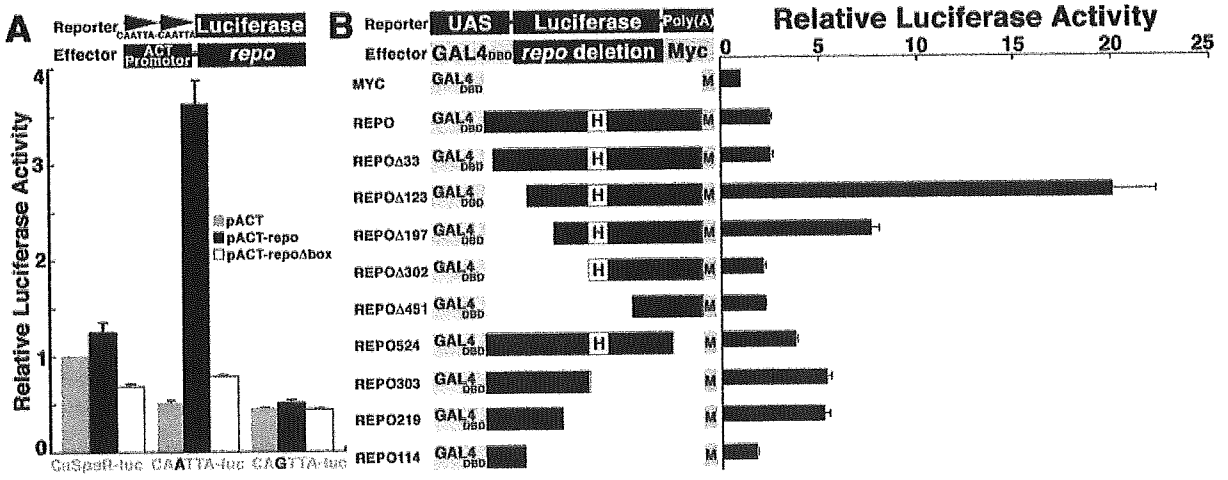

Fig. 1. REPO is a transcriptional activator. (A) REPO activates transcription through a homeodomain-binding site. The effector constructs express REPO (pACT-repo) or REPO lacking its homeodomain (REPOΔbox, pACT-repoΔbox), driven by the *Drosophila Act5C* promoter. The luciferase reporter contains two copies of CAATTA (CAATTA-luc) or CAGTTA (CAGTTA-luc) motifs placed upstream of the *hsp70* minimal promoter. The CaSpeR-luc reporter has a luciferase gene with the *hsp70* minimal promoter alone. Three luciferase reporter plasmids were each transfected into S2 cells with pACT-repo (black column), pACT-repoΔbox (white column) or vector (pACT) alone (gray column). The amount of luciferase activity generated by the co-transfection of CaSpeR-luc and pACT was defined as 1. (B) REPO contains multiple transcriptional activation domains. Various regions of REPO were fused to the GAL4 DNA-binding domain (DBD) and tagged with three copies of the Myc epitope at their C terminus. The reporter was the luciferase gene placed downstream of the *hsp70* TATA and five copies of UAS (GAL4-binding sites). The luciferase activity obtained with each effector is presented as the relative value compared with the value obtained with GAL4-DBD alone. The expression of the GAL4 fusion proteins was confirmed by western blot analysis of whole-cell extracts using a monoclonal antibody directed against the Myc-tag. H, homeodomain; M, Myc tag.

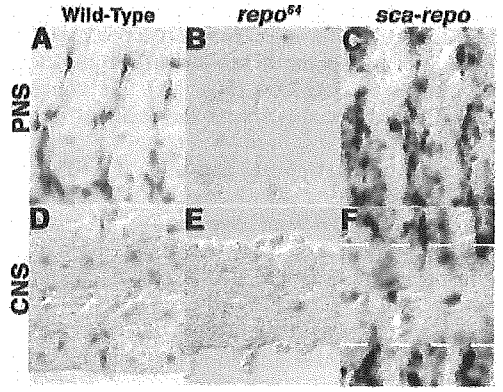

Fig. 2. REPO activates the expression of the *ftz* HDS reporter gene in glial cells. (A,D) Wild-type embryos; (B,E) *repo* mutant embryos; (C,F) ectopic expression of *repo* in *scabrous*-GAL4/UAS-*repo*. (A-C) Stage 16 embryonic PNS preparations. (A) In wild-type embryos, expression of the *ftz* HDS reporter gene is detected in glial cells of the peripheral nervous system, including the support cells of the bipolar dendritic neuron, peripheral glia, ligament cells of the chordotonal organ and exit glia. (B) In the *repo* mutant, the expression of the *ftz* HDS reporter gene in the PNS was absent or dramatically reduced. (C) In response to the ectopic expression of REPO, many cells in the dorsal epidermis expressed the *ftz* HDS reporter. (D-F) Dissected stage 16 embryonic CNS preparations. (D) In the wild-type CNS, the *ftz* HDS reporter gene is expressed in a subset of longitudinal glia, the A glia, B glia and intersegmental nerve root glia. (E) In the *repo* mutant, the expression of the *ftz* HDS reporter gene in the CNS glia was greatly reduced, as it was in the PNS. (F) Ectopic expression of REPO did not induce ectopic expression of the *ftz* HDS reporter gene within the CNS, although many cells showed ectopic expression in the periphery. Anterior is leftwards.

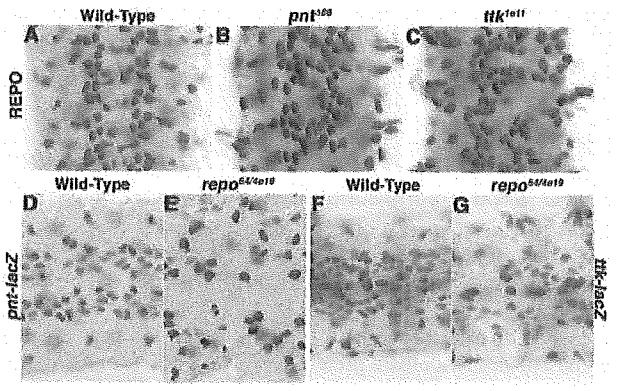

Fig. 3. *repo*, *pointed* and *ttk* are expressed independent of each other. (A-C) The expression of *repo* was demonstrated using a REPO antibody. (A) In wild-type embryos, REPO is expressed in all CNS glia except the midline glia. REPO-positive glial cells were still present in $pnt^{\Delta 88}$ (B) or ttk^{1e11} (C) mutant embryos. (D) A *pointed* enhancer-trap strain (pnt^{rM254}) expressed β-galactosidase in CNS glia that expressed REPO. (E) pnt^{rM254} was expressed in CNS glia in *repo* mutant embryos. (F) A *ttk* enhancer-trap strain (ttk^{0219}) expressed β-galactosidase in all CNS glia, in wild-type (F) as well as *repo* (G) mutant embryos. Anterior is upwards in A-C and leftwards in D-G.

the *ftz* HDS *lacZ* reporter gene in the *repo* mutant background. This reporter gene (2x21F) carries two copies of a 21 mer containing the CAATTA motif, and is expressed in all glial cells in the PNS and a subset of CNS glia (Nelson and Laughon, 1993) (Fig. 2A,D). The glial expression of the *ftz* HDS reporter gene in the PNS overlaps precisely with REPO-expressing cells. The loss of *repo* function abolished *lacZ* expression in both the PNS and CNS glia (Fig. 2B,E), even though glial cells are still present in stage 16 *repo* mutant embryos (Halter et al., 1995). Expression of the *ftz* HDS reporter gene in the antenno-maxillary complex and posterior spiracles, where REPO is not expressed, was unaffected in *repo* mutants (data not shown). These results indicate that REPO acts through the CAATTA site to drive transcription in glial cells.

To address whether REPO is sufficient to activate transcription of the *ftz* HDS reporter gene, we expressed REPO ectopically in the presumptive ventral neurogenic region and the dorsal epidermis. Such embryos expressed *lacZ* in non-glial cells within the dorsal epidermis, adjacent to the PNS (Fig. 2C). Thus, REPO is sufficient for the expression of the *ftz* HDS reporter gene in specific cellular contexts.

Although ectopic REPO induced the appearance of many non-glial *lacZ*-expressing cells in the dorsal epidermis, cells within the CNS did not respond to ectopic REPO. In fact, even in the wild-type background, not all REPO-positive glia in the CNS expressed the *ftz* HDS reporter. This suggests that the mechanism by which REPO regulates transcription may be different in the CNS from the one for peripheral glia. One possible scenario is that the functions of REPO in the CNS requires cooperation with one or more other factors, and that these interactions preclude REPO from acting through the CAATTA motif. TTK69 and PNTP1 are good candidates for such co-factors, because *ttk* and *pointed* are both required for the development of CNS glial cells (Klaes et al., 1994; Giesen et al., 1997). Although *repo*, *ttk* and *pointed* are expressed in overlapping subsets of CNS glial cells, their expression is mutually independent; REPO continued to be expressed in the *ttk* or *pointed* mutant background, and *lacZ* expression levels in enhancer-trap lines of *ttk* or *pointed* were unaffected in *repo* mutant embryos (Fig. 3). Moreover, ectopic expression of REPO in the entire neuroectoderm did not increase the expression of *pointed* P1 mRNA or TTK69, nor did ectopic expression of either TTK69 or PNTP1 affect REPO expression (data not shown). All three genes are most probably regulated independently, downstream of the glial determinant GCM (Hosoya et al., 1995; Jones et al., 1995; Giesen et al., 1997).

REPO cooperates with TTK69 to induce expression of the glial marker M84 in the CNS

To study how REPO controls its target genes in the CNS, we analyzed the regulation of a glial enhancer trap marker M84 (Klämbt and Goodman, 1991), whose expression requires *repo* function (Halter et al., 1995) (Fig. 4B). The M84 strain labels small subsets of glial cells in the CNS (Ito et al., 1995). All M84-expressing glia also expressed REPO and TTK69, but some were negative for PNTP1 expression (data not shown). Although M84 was expressed normally in *pointed* mutants, in *ttk* mutant embryos a prominent decrease in the level of *lacZ* expression was seen in M84-positive glial cells (Fig. 4C,D).

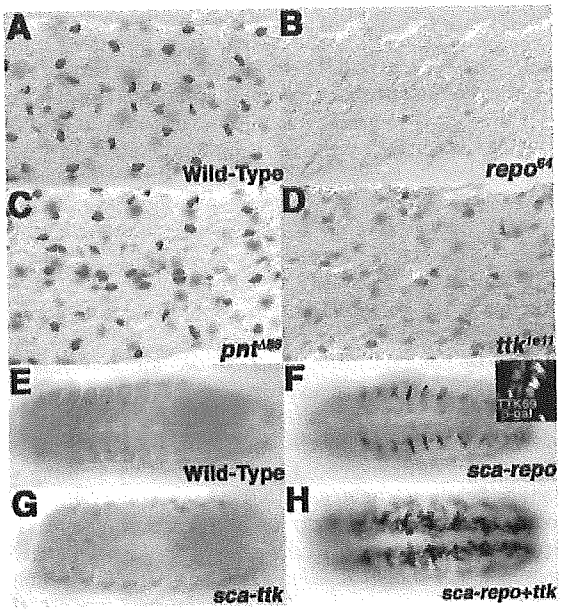

Fig. 4. Cooperative action of REPO and TTK69 activates the glial expression of the M84 marker. (A-D) CNS of stage 16 embryos carrying the M84 glial marker. (A) In wild-type embryos, M84 β-galactosidase expression could be detected in the subperineurial glia and channel glia. (B) In *repo* mutant embryos, M84 expression was absent or dramatically reduced. (C) *pointed* mutant embryos expressed the M84 marker at normal levels, although the arrangement of the glial cells was irregular. (D) In *ttk* mutant embryos, the expression level of M84 was lower than in wild type. (E-H) Ventral views of stage 12 embryos carrying the M84 glial marker. In wild-type embryos, few cells expressed the M84 marker at stage 13 (E). Ectopic expression of REPO induced additional cells to express the M84 marker (F), whereas TTK69 had no effect (G). Co-expression of REPO and TTK69 had a synergistic effect on the activation of the M84 marker (H). (F, inset) Supernumerary M84-positive cells induced by the ectopic expression of REPO also expressed endogenous TTK69. The ectopically expressed M84 marker is shown in magenta and TTK69 is shown in green. An overlay of both colors appears as white. The majority of ectopic M84-positive cells were located in the epidermis, which normally do not express this marker. The number of M84-positive epidermal cells per hemisegment were 10.2±5.8 for REPO misexpression and 32.6±9.7 for co-expression of REPO and TTK69 (n=23). Anterior is leftwards.

Thus, M84 is a suitable glial marker to examine the cooperation between REPO and TTK69.

We examined the effect of the ectopic expression of REPO and TTK69 on M84 expression. The expression of REPO in the neuroectoderm resulted in a precocious expression of the M84 marker in the CNS. In normal embryos, the majority of M84-positive cells appear only in stage 13 among REPO-positive cells that probably differentiate into subperineurial glia and channel glia (data not shown) (Halter et al., 1995; Ito et al., 1995). When REPO was expressed ectopically throughout the entire neuroectoderm, M84-positive cells appeared at stage 12 at the lateral edge of the CNS (Fig. 4F) and could be observed at stage 16 in cells that do not normally express REPO (data not shown). Many of the cells that expressed *lacZ* ectopically and precociously were located in the epidermis. Such cells were also positive for TTK69 (Fig. 4F, inset), suggesting that TTK69 is a prerequisite for REPO to induce expression of the glial marker M84. Although the forced expression of TTK69 alone had no effect on the expression of M84, the co-expression of REPO and TTK69 caused a greater than threefold increase in the number of M84-expressing cells, compared with the expression of REPO alone (Fig. 4G,H). Thus REPO cooperates with TTK69 to induce expression of the glial marker M84.

Expression of *loco-c1* depends on both REPO and PNTP1

Although results presented above suggest a synergistic action of REPO and TTK69 in transcriptional activation, we cannot determine whether REPO and TTK69 act cooperatively on the same target gene, because the gene responsible for M84 is not known. We thus studied the regulation of the gene *loco*, which encodes a member of the family of Regulator of G-protein Signaling (Granderath et al., 1999; Granderath et al., 2000). The *loco* function is required for glial morphogenesis, and *loco-c1*, an isoform of *loco*, is expressed specifically in REPO-positive glial cells, which also express PNTP1 (Granderath et al., 1999; Granderath et al., 2000). The expression of a *loco* reporter gene carrying a glial enhancer element of *loco* (Rrk; Fig. 5D) requires PNTP1 function, as well as an Ets-binding site located within this glial enhancer element (Granderath et al., 2000). Although this *loco*-reporter gene is expressed normally in *repo* mutants (Granderath et al., 2000), we found that in stage 16 *repo* mutant embryos *loco-c1* mRNA was reduced to undetectable levels (Fig. 5B,C), whereas such embryos exhibit robust expression of a *pnt-lacZ* reporter gene in glial cells (Fig. 3E). It is thus likely that proper expression of *loco* depends on both *pointed* and *repo* function.

To test whether REPO acts directly on the *loco* promoter, we focused on a 0.7 kb *loco* promoter fragment (AEE), which partially overlaps with the glial enhancer fragment (Fig. 5D). AEE contains two CAATTA motifs, one of which is located outside the glial enhancer element (Rrk) used by Granderath et al. (Granderath et al., 2000). We constructed luciferase reporter genes that carry either wild type AEE or AEE with single base changes in the CAATTA motifs (AEE-*luc* and AEE*-*luc*, Fig. 5D), and introduced them into S2 cells. Co-transfection of the AEE-*luc* reporter with a REPO expression plasmid caused a 45-fold increase in luciferase activity compared with co-transfection with the vector alone, whereas single base changes in the CAATTA motifs caused a fivefold drop in REPO-dependent activation (Fig. 5E). These results suggest that REPO directly regulates *loco* *trans*cription through the CAATTA motif.

As *loco* expression requires both *repo* and *pointed*, we studied the effects of co-expressing REPO and PNTP1. The *loco* genomic fragment in AEE-*luc* reporter was extended 0.7 kb in the 5′ direction, to include the Ets-binding site through which PNTP1 acts (AES-*luc*, Fig. 5D). In S2 cells transfected with a plasmid that directs REPO expression, a fivefold increase in this reporter gene expression was achieved, compared with transfection with the vector alone (Fig. 5F). Although the expression of PNTP1 had little effect on the reporter gene, co-expression of PNTP1 with REPO resulted in a significantly higher level of reporter expression than the expression of REPO alone (Fig. 5F). Taken together with the