including the VAS value for fatigue, no statistical differences were observed between the 2 sessions. We analyzed baseline-adjusted net changes in order to evaluate the effects of mental fatigue on all parameters and in order to control for intra-individual variability of baseline values over 4 weeks; and these changes after 8 hr of relaxation or mental fatigue and those assessed the next morning are shown in Tables 3 and 4. Two-way ANOVA for repeated measures of the VAS value for fatigue revealed that the baseline-adjusted net change in the value after the fatigue session was significantly higher relative to that after the relaxation session ($F = 17.11$; $df = 1, 16$; $P < 0.001$). The

Table 3. *Baseline-adjusted net changes in various parameters after relaxation or fatigue session and the next morning*

	8 hr		Next morning	
	Relaxation	Fatigue	Relaxation	Fatigue
VAS	5.6 ± 24.7	47.4 ± 21.3[a]	−8.6 ± 16.0	16.7 ± 20.0
Total protein, g/l	−3.9 ± 3.4	−0.6 ± 5.3	−3.8 ± 2.6	−4.8 ± 4.5
Albumin, g/l	−2.6 ± 1.9	−0.8 ± 3.5	−2.3 ± 1.1	−4.4 ± 2.8
Plasma glucose, mmol/l	−0.32 ± 0.30	−0.25 ± 0.43	0.20 ± 0.35	0.22 ± 0.27
Total cholesterol, mmol/l	−0.29 ± 0.17	−0.07 ± 0.39	−0.25 ± 0.13	−0.36 ± 0.39
HDL cholesterol, mmol/l	−0.11 ± 0.06	−0.02 ± 0.18	−0.12 ± 0.09	−0.16 ± 0.15
Triacylglycerol, mmol/l	0.14 ± 0.20	0.03 ± 0.16	0.29 ± 0.29	0.18 ± 0.39
Creatine phosphokinase, IU/l	−5.8 ± 12.1	−12.1 ± 26.5	16.6 ± 78.1	−9.9 ± 29.8
Cortisol, nmol/l	−153.9 ± 135.0	−171.7 ± 101.4	90.1 ± 129.9	105.8 ± 135.5

VAS Visual analogue scale; *HDL* high density lipoprotein.
Data are presented as the mean ± SD.
[a] $P < 0.01$, significantly different from the corresponding values of the relaxation session (2-way ANOVA for repeated measures, followed by paired *t*-test with Bonferroni correction).

Table 4. *Baseline-adjusted net changes in plasma amino acid levels after relaxation or fatigue session and the next morning*

	8 hr		Next morning	
	Relaxation	Fatigue	Relaxation	Fatigue
Valine, mmol/l (%)	62.7 ± 35.9 (32.5)	17.4 ± 37.1 (10.4)[a]	24.5 ± 35.1 (14.5)	8.3 ± 31.1 (6.2)
Leucine, mmol/l (%)	48.0 ± 29.2 (48.0)	−3.3 ± 24.1 (−0.5)[a]	20.2 ± 24.2 (22.8)	2.4 ± 16.1 (4.4)
Isoleucine, mmol/l (%)	36.9 ± 20.2 (67.6)	9.4 ± 17.9 (19.6)[b]	19.6 ± 16.7 (38.7)	10.7 ± 6.4 (20.1)
BCAA, mmol/l (%)	147.6 ± 81.9 (41.8)	23.5 ± 75.9 (8.4)[a]	64.4 ± 74.3 (20.4)	21.5 ± 51.4 (7.7)
Tyrosine, mmol/l (%)	16.2 ± 14.6 (33.9)	−2.4 ± 10.5 (−2.4)[b]	13.1 ± 13.2 (26.3)	9.9 ± 6.9 (17.7)
Phenylalanine, mmol/l (%)	14.4 ± 8.1 (27.4)	4.5 ± 8.2 (9.5)	5.6 ± 6.0 (11.2)	−0.6 ± 7.3 (0)
Tryptophan, mmol/l (%)	9.5 ± 20.7 (20.5)	−4.9 ± 5.2 (−7.8)	3.5 ± 19.2 (9.6)	−2.3 ± 5.7 (−3.5)
Cysteine, mmol/l (%)	3.0 ± 3.7 (10.4)	−3.8 ± 3.2 (−9.1)[a]	7.5 ± 3.2 (25.3)	−2.0 ± 1.9 (−5.0)[c]
Methionine, mmol/l (%)	14.6 ± 11.0 (65.4)	−1.8 ± 6.9 (−4.5)[a]	6.1 ± 7.4 (28.5)	0.6 ± 3.6 (3.7)
Lysine, mmol/l (%)	64.1 ± 36.5 (38.1)	2.8 ± 35.5 (2.7)[b]	27.0 ± 29.1 (16.9)	−15.3 ± 20.6 (−6.9)[b]
Arginine, mmol/l (%)	38.2 ± 19.2 (59.3)	7.6 ± 14.5 (11.3)[b]	16.5 ± 13.2 (27.8)	5.4 ± 4.9 (7.7)
Histidine, mmol/l (%)	9.0 ± 6.1 (12.5)	0.6 ± 7.9 (1.3)	5.2 ± 6.7 (7.7)	−4.8 ± 6.6 (−5.5)
Serine, mmol/l (%)	10.0 ± 45.4 (14.3)	0.9 ± 13.6 (0)	7.6 ± 42.7 (11.4)	4.9 ± 9.9 (3.8)
Threonine, mmol/l (%)	17.2 ± 22.8 (14.4)	−6.0 ± 22.3 (−3.8)	19.2 ± 35.5 (17.5)	3.9 ± 18.4 (4.5)
Asparagine, mmol/l (%)	11.1 ± 11.0 (28.7)	5.8 ± 8.6 (12.5)	6.3 ± 10.6 (17.0)	2.2 ± 5.7 (4.8)
Aspartate, mmol/l (%)	N.D.	N.D.	N.D.	N.D.
Glutamine, mmol/l (%)	63.5 ± 35.5 (11.1)	20.6 ± 55.9 (4.2)	39.5 ± 42.7 (6.9)	−10.5 ± 33.1 (−1.5)
Glutamate, mmol/l (%)	−0.4 ± 5.5 (−2.8)	−2.7 ± 9.8 (−7.7)	3.4 ± 8.6 (16.6)	4.9 ± 9.7 (16.1)
Glycine, mmol/l (%)	32.3 ± 117.0 (25.6)	15.7 ± 31.8 (5.9)	24.7 ± 119.0 (22.2)	19.3 ± 18.1 (8.1)
Alanine, mmol/l (%)	75.9 ± 51.1 (27.6)	33.6 ± 48.1 (13.2)	33.4 ± 63.9 (13.5)	40.5 ± 37.7 (13.6)
Proline, mmol/l (%)	32.3 ± 29.3 (27.0)	23.2 ± 18.5 (20.9)	14.0 ± 34.5 (15.1)	19.7 ± 16.5 (18.2)

BCAA Branched-chain amino acids; *N.D.* not detected.
Data are presented as the mean ± SD.
The percent change in mean plasma level of amino acids after the 8-hr session or the next morning relative to that of the baseline level is given in parentheses.
[a] $P < 0.05$, [c] $P < 0.01$, significantly different from the corresponding values of the relaxation session (2-way ANOVA for repeated measures, followed by paired *t*-test with Bonferroni correction). [b] $P < 0.1$, different from the corresponding values of the relaxation session (2-way ANOVA for repeated measures, followed by paired *t*-test with Bonferroni correction).

VAS value for the next morning after the fatigue session tended to show recovery. There were no significant differences in baseline-adjusted net changes in plasma glucose, serum total protein, albumin, total cholesterol, high density lipoprotein, cholesterol, triacylglycerol, creatine phosphokinase, and cortisol levels between the 2 groups just after the sessions or the next morning. ANOVA analyses also revealed main effects of the session on plasma valine ($F=4.51$; $df=1$, 16; $P=0.049$), leucine ($F=12.94$; $df=1$, 16; $P=0.002$), isoleucine ($F=9.24$; $df=1$, 16; $P=0.008$), BCAA ($F=8.39$; $df=1$, 16; $P=0.011$), tyrosine ($F=5.68$; $df=1$, 16; $P=0.030$), cysteine ($F=38.98$; $df=1$, 16; $P<0.001$), methionine ($F=11.67$; $df=1$, 16; $P=0.004$), lysine ($F=15.53$; $df=1$, 16; $P=0.001$), and arginine ($F=15.39$; $df=1$, 16; $P=0.001$) levels. Baseline-adjusted net changes in plasma valine, leucine, BCAA, cysteine, and methionine levels after the fatigue session were significantly lower than those after the relaxation session. In addition, baseline-adjusted net changes in plasma isoleucine, tyrosine, lysine, and arginine levels after the fatigue session had a trend toward lower than those after the relaxation session. Baseline-adjusted net change in plasma cysteine level for the next morning following the fatigue session was significantly lower, and that in plasma arginine level had a trend toward lower than those following the relaxation session.

Discussion

Although there are a number of reports in the literature about the relationship between physical fatigue and plasma levels of amino acids (Blomstrand et al., 1991; Blomstrand, 2001; Lehmann et al., 1995), the influence of mental fatigue on these levels remains unknown. Plasma amino acid levels are thought to be influenced by brain activities; e.g., a molecular imaging study showed that the plasma level of tyrosine, which is a precursor amino acid of dopamine, was correlated with the extent of neurotransmission (Leyton et al., 2004). Therefore, in order to investigate the relationship between amino acid metabolism and mental fatigue, we measured the plasma levels of all 20 amino acids before and after relaxation and fatigue sessions. This is the first study to show that the concentrations of 8 plasma amino acids were significantly or trend toward changed by mental fatigue.

The baseline-adjusted net change in the VAS value after the fatigue session was approximately 8-times higher relative to that after the relaxation session, indicating that the subjects had a fatigue sensation by performing the series of mental tasks during the fatigue session. There was a high degree of variability in the VAS values given by the individuals during the relaxation and fatigue sessions. Since subjective fatigue sensation is influenced not only by individual's fatigue level, but also by many factors (e.g., motivation and sensitivity for fatigue), such a high degree of variability in the subjective fatigue score might happen. There were no significant differences in baseline-adjusted net changes in creatine phosphokinase level between fatigue and relaxation sessions. These results suggest that the subjects performed continual mental tasks without marked physical load. As for stress load, we have no available data about saliva cortisol level, self report-scale of stress, skin conductance, or heart rate variability. Therefore, results of our study seem to reflect mental fatigue and/or stress associated with performing the tasks.

The baseline-adjusted net changes in the plasma levels of valine, leucine, cysteine, and methionine levels after the fatigue session were significantly lower and those in the plasma isoleucine, tyrosine, lysine, and arginine had a trend toward lower than those after the relaxation session. Blood levels of other parameters including total protein, albumin, glucose, and total cholesterol did not show any differences between the 2 sessions. These results indicate that consumption of the above 8 amino acids was accelerated when the subjects performed the mental tasks. In contrast, the plasma levels of glycine, proline, asparagine, aspartate, and glutamate, all of which show low potential extraction rates through the blood–brain barrier (Oldendorf, 1971), were not affected by the mental task. These results suggest that the consumption of the 8 amino acids was caused by the brain activities.

Besides being used for protein synthesis, amino acids are also involved in various metabolic activities in the brain: e.g., BCAA are transaminated to α-ketoglutarate for the synthesis of glutamate (Yudkoff, 1997); tyrosine is utilized for the synthesis of dopamine (Wurtman et al., 1974); cysteine is used for the synthesis of glutathione and taurine (Kranich et al., 1998; Parcell, 2002); and arginine is required for nitric oxide production and for creatine synthesis (Bredt and Snyder, 1994; Wyss and Kaddurah, 2000). It has been reported that in animals subjected to stress for several hours the release and synthesis of glutamate (Gilad et al., 1990; Moghaddam, 1993) and dopamine (Imperato et al., 1991; Nakahara and Nakamura, 1999) were enhanced in their brain. These findings suggest that mental load accelerates the consumption of neurotransmitters. Accordingly, utilization of tyrosine may be increased for the synthesis of catecholamines; and therefore the supply of tyrosine through phenylalanine by liver phenylalanine hydroxylase may proceed. Although we did not measure plasma free-

tryptophan level, change of plasma tryptophan level after the fatigue session had a trend toward lower than that after the relaxation session ($P=0.056$, paired t-test without Bonfferoni correction). Increase in the utilization of tryptophan for the synthesis of serotonin may also happen. In addition, prolonged psychological stresses induced an increase in the level of thiobarbituric acid reactive substance, an index of lipid preoxidation activity, in the brain (Matsumoto et al., 1999), thus suggesting that oxidative stress is brought about by mental load. Thus, utilization of cysteine and arginine may be increased for the synthesis of antioxidants. Taken together, a possible interpretation of these results might be that mental fatigue could facilitate utilization of amino acids for the synthesis of neurotransmitters or antioxidants in the central nervous system.

On the morning after the fatigue session, baseline-adjusted net changes in plasma cysteine and lysine levels remained lower as compared with those for the relaxation session, thus suggesting prolonged effects of mental fatigue on the plasma levels of these particular amino acids. We speculate that the consumption of the amino acids that are precursors of antioxidants, was accelerated during the recovery phase from mental fatigue and that antioxidants are essential for the recovery from mental fatigue.

There are some limitations to our study, such as the small number of subjects and study design. To generalize our results, the study with a larger number of subjects is essential. However, not only decreased levels of plasma amino acids (valine, leucine, isoleucine, tyrosine, cysteine, methionine, lysine, and arginine) were shown just after the mental tasks, but also those of plasma cysteine and lysine were shown in the next morning relative to the relaxation tasks. We believe the validity of our results to some extent.

The plasma levels of these amino acids levels might thus be good biomarkers of mental fatigue or at least mental task-induced fatigue. Supplementation of the diet with the above amino acids may be useful for lowering mental fatigue and for facilitating recovery from it. We now plan to examine the uptake of radiolabeled amino acids into the brain by using positron emission tomography (PET) as well as the effects of amino acid administration on mental fatigue and the fatigue sensation.

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References

Bailey SP, Davis JM, Ahlborn EN (1992) Effect of increased brain serotonergic activity on endurance performance in the rat. Acta Physiol Scand 145: 75–76

Bailey SP, Davis JM, Ahlborn EN (1993) Neuroendocrine and substrate responses to altered brain 5-HT activity during prolonged exercise to fatigue. J Appl Physiol 74: 3006–3012

Blomstrand E (2001) Amino acids and central fatigue. Amino Acids 20: 25–34

Blomstrand E, Perrett D, Parry-Billings M, Newsholme EA (1989) Effect of sustained exercise on plasma amino acid concentrations and on 5-hydroxytryptamine metabolism in six different brain regions of the rat. Acta Physiol Scand 136: 473–481

Blomstrand E, Hassmen P, Ekblom B, Newsholme EA (1991) Administration of branched-chain amino acids during sustained exercise-effects on performance and on plasma concentration of some amino acids. Eur J Appl Physiol 63: 83–88

Bredt DS, Snyder SH (1994) Nitric oxide: a physiologic messenger molecule. Annu Rev Biochem 63: 175–195

Chaouloff F, Laude D, Elghozi JL (1989) Physical exercise: evidence for differential consequences of tryptophan on 5-HT synthesis and metabolism in central serotonergic cell bodies and terminals. J Neural Transm 78: 121–130

Chaudhuri A, Behan PO (2000) Fatigue and basal ganglia. J Neurol Sci 179: 34–42

Chaudhuri A, Behan PO (2004) Fatigue in neurological disorders. Lancet 363: 978–988

Ellenbogen MA, Young SN, Dean P, Palmour RM, Benkelfat C (1996) Mood response to acute tryptophan depletion in healthy volunteers: sex differences and temporal stability. Neuropsychopharmacology 15: 465–474

Fitts RH (1994) Cellular mechanisms of muscle fatigue. Physiol Rev 74: 49–94

Gilad GM, Gilad VH, Wyatt RJ, Tizabi Y (1990) Region-selective stress-induced increase of glutamate uptake and release in rat forebrain. Brain Res 525: 335–338

Imperato A, Puglisi-Allegra S, Casolini P, Angelucci L (1991) Changes in brain dopamine and acetylcholine release during and following stress are independent of the pituitary-adrenocortical axis. Brain Res 538: 111–117

Kajimoto O, Shimizu A, Takahashi T, Iwase M, Takahashi R, Kuratsune H, Watanabe Y (2007) ATMT: a computer-assisted system for assessment of fatigue -Development of advanced trail making test for evaluating mental function. J Chron Fatigue Syndrome (in press)

Kranich O, Dringen R, Sandberg M, Hamprecht B (1998) Utilization of cysteine and cysteine precursors for the synthesis of glutathione in astroglial cultures: preference for cystine. Glia 22: 11–18

Lehmann M, Huonker M, Dimeo F, Heinz N, Gastmann U, Treis N, Steinacker JM, Keul J, Kajewski R, Haussinger D (1995) Serum amino acid concentration in nine athletes before and after the 1993 Colmar Ultra Triathlon. Int J Sports Med 16: 155–159

Leyton M, Dagher A, Boileau I, Casey K, Baker GB, Diksic M, Gunn R, Young SN, Benkelfat C (2004) Decreasing amphetamine-induced dopamine release by acute phenylalanine/tyrosine depletion: a PET/[^{11}C]raclopride study in healthy men. Neuropsychopharmacology 29: 427–432

Matsumoto K, Yobimoto K, Huong NT, Abdel-Fattah M, Van HT, Watanabe H (1999) Psychological stress-induced enhancement of brain lipid peroxidation via nitric oxide systems and its modulation by anxiolytic and anxiogenic drugs in mice. Brain Res 839: 74–84

Moghaddam B (1993) Stress preferentially increases extraneuronal levels of excitatory amino acids in the prefrontal cortex: comparison to hippocampus and basal ganglia. J Neurochem 60: 1650–1657

Nakahara D, Nakamura M (1999) Differential effect of immobilization stress on in vivo synthesis rate of monoamines in medial prefrontal cortex and nucleus accumbens of conscious rats. Synapse 32: 238–242

Oldendorf WH (1971) Brain uptake of radiolabeled amino acids, amines, and hexoses after arterial injection. Am J Physiol 221: 1629–1639

Parcell S (2002) Sulfur in human nutrition and applications in medicine. Altern Med Rev 7: 22–44

Pardridge WM (1977) Kinetics of competitive inhibition of neutral amino acid transport across the blood–brain barrier. J Neurochem 28: 103–108

Reitan RM (1955) The relation of the trail making test to organic brain damage. J Consult Psychol 19: 393–394

Wurtman RJ, Larin F, Mostafapour S, Fernstrom JD (1974) Brain catechol synthesis: control by brain tyrosine concentration. Science 185: 183–184

Wyss M, Kaddurah DR (2000) Creatine and creatinine metabolism. Physiol Rev 80: 1107–1213

Yamamoto K (1992) Analysis of cognitive function and regional cerebral blood flow in Parkinson's disease by ^{123}I-IMP SPECT (in Japanese). Rinsho Shinkeigaku 32: 1–7

Yoshiuchi K, Nomura S, Ando K, Ohtake T, Shimosawa T, Kumano H, Kuboki T, Suematsu H, Fujita T (1997) Hemodynamic and endocrine responsiveness to mental arithmetic task and mirror drawing test in patients with essential hypertension. Am J Hypertens 10: 243–249

Yudkoff M (1997) Brain metabolism of branched-chain amino acids. Glia 21: 92–98

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Multi-directional differentiation of doublecortin- and NG2-immunopositive progenitor cells in the adult rat neocortex *in vivo*

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Abstract

In the adult mammalian brain, multipotent stem or progenitor cells involved in reproduction of neurons and glial cells have been well investigated only in very restricted regions; the subventricular zone of the lateral ventricle and the dentate gyrus in the hippocampal formation. In the neocortex, a series of *in vitro* studies has suggested the possible existence of neural progenitor cells possessing neurogenic and/or gliogenic potential in adult mammals. However, the cellular properties of the cortical progenitor cells *in vivo* have not been fully elucidated. Using 5′-bromodeoxyuridine labeling and immunohistochemical analysis of cell differentiation markers, we found that a subpopulation of NG2-immunopositive cells co-expressing doublecortin (DCX), an immature neuron marker, ubiquitously reside in the adult rat neocortex. Furthermore, these cells are the major population of proliferating cells in the region. The DCX(+)/NG2(+) cells reproduced the same daughter cells, or differentiated into DCX(+)/NG2(–) (approximately 1%) or DCX(–)/NG2(+) (approximately 10%) cells within 2 weeks after cell division. The DCX(+)/NG2(–) cells were also immunopositive for TUC-4, a neuronal linage marker, suggesting that these cells were committed to neuronal cell differentiation, whereas the DCX(–)/NG2(+) cells showed faint immunoreactivity for glutathione S-transferase (GST)-pi, an oligodendrocyte lineage marker, in the cytoplasm, suggesting glial cell lineage, and thereafter the cells differentiated into NG2(–)/GST-pi(+) mature oligodendrocytes after a further 2 weeks. These findings indicate that DCX(+)/NG2(+) cells ubiquitously exist as 'multipotent progenitor cells' in the neocortex of adult rats.

Introduction

In adult mammals, continuous reproduction of neurons is known in two restricted regions: the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone of the hippocampal dentate gyrus (Cameron *et al.*, 1993; Lois & Alvarez-Buylla, 1993). These neurogenic regions contain neural progenitor cells that possess proliferative potential including self-reproduction, and can generate a wide variety of neural cells including neurons, astrocytes and oligodendrocytes, *in vitro* (Morshead *et al.*, 1994; Gage *et al.*, 1995; Gritti *et al.*, 1999). The neural progenitor cells in the neurogenic regions *in vivo* include glial fibrillary acidic protein-immunopositive [GFAP(+)] cells and the polysialyated form of neural cell adhesion molecule-immunopositive [PSA-NCAM(+)] cells, which are derived from GFAP(+) cells (Doetsch *et al.*, 1999, 2002; Seri *et al.*, 2001). Some neural progenitor cells in the SVZ have also been reported to express NG2 chondroitin sulfate proteoglycan (Belachew *et al.*, 2003).

Observations that cells isolated from the adult rat cerebral cortex self-renewed and generated both neurons and glial cells under cultured conditions have implied that the cerebral cortex also includes neural progenitor cells (Palmer *et al.*, 1999). However, very little is known about the cellular properties of cortical progenitor cells *in vivo*. Recently, NG2(+) cells isolated from the cerebral gray matter of early postnatal mice were shown to form neurospheres and gave rise to neurons, as well as astrocytes and oligodendrocytes *in vitro* (Belachew *et al.*, 2003). Moreover, NG2(+) cells were found to be the major proliferating cell population in the cerebral cortex of adult rats *in vivo* (Dawson *et al.*, 2003; Tamura *et al.*, 2004). These reports indicate that NG2(+) cells may be a candidate for neural progenitor cells in the adult cortex. However, it remains unclear whether all NG2(+) cells are cortical progenitor cells and whether the cells have multipotent activities *in vivo*. Indeed, it has been reported that only a small fraction of cortical NG2(+) cells isolated from early postnatal mice

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are immunopositive for Tuj1 and HuC/D, markers for immature neurons (Chittajallu *et al.*, 2004). Moreover, different subtypes of NG2(+) cells have been identified in the gray matter of adult rodent brain on the basis of morphological criteria (Nishiyama *et al.*, 1999) and physiological properties (Chittajallu *et al.*, 2004). These observations suggest that NG2(+) cells are composed of heterogeneous cellular populations.

In the present study, we identified progenitor cells that express doublecortin (DCX), an immature neuron marker, among NG2(+) cells in the neocortex of adult rats *in vivo*. Furthermore, we examined whether these cells have multipotent activity by tracing their differentiation.

Materials and methods

Animals and BrdU injections

Adult male Wistar rats (SLC, Hamamatsu, Japan; 250–350 g body weight) were used. All experimental protocols were approved by the Ethics Committee on Animal Care and Use, Kansai Medical University, and were performed in accordance with the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985). For labeling of proliferating cells, adult rats ($n = 15$) were intraperitoneally injected with 5-bromodeoxyuridine (BrdU) at 50 mg/kg body weight. Each animal underwent a single injection of BrdU.

Immunohistochemistry

Animals were deeply anesthetized with diethyl ether and perfused transcardially with 4% formaldehyde buffered with 0.1 M phosphate-buffered saline (PBS; pH 7.4) at 2 h ($n = 5$), 14 days ($n = 5$) and 28 days ($n = 5$) after injection of BrdU (Sigma, St Louis, MO, USA). Brains were removed, postfixed overnight at 4 °C in 4% formaldehyde buffered with 0.1 M PBS and then immersed in 20% (w/v) sucrose solution. Coronal brain sections (30 μm thickness) were prepared using a cryostat and collected as free-floating sections. For detection of BrdU incorporation, brain sections were preincubated in 50% formamide/2 × standard sodium citrate (SSC) for 2 h at 65 °C, incubated in 2 N HCl for 30 min at 37 °C, rinsed in 0.1 M boric acid (pH 8.5) for 10 min at 25 °C, and then washed with 0.3% Triton X-100 in PBS (PBST). For multiplex-immunostaining, coronal sections were incubated with several primary antibodies for 12–36 h at 4 °C. The primary antibodies used in this study were: monoclonal rat anti-BrdU IgG (1 : 10, Oxford Biotechnology, Oxford, UK); polyclonal rabbit anti-Ki67 IgG (1 : 1000, NovoCastra, Newcastle, UK); polyclonal goat anti-DCX (C-18) IgG (1 : 100, Santa Cruz Biotechnology, Santa Cruz, USA); monoclonal mouse anti-PSA-NCAM IgM (1 : 200, Chemicon, Temecula, CA, USA); polyclonal rabbit anti-TUC-4 protein IgG (1 : 1000, Chemicon); monoclonal mouse anti-neuronal nuclei (NeuN) IgG (1 : 200, Chemicon); monoclonal mouse anti-NG2 IgG (1 : 200, Chemicon); polyclonal rabbit anti-NG2 IgG (1 : 200, Chemicon); polyclonal rabbit anti-glutathione S-transferase (GST)-pi IgG (1 : 500, Medical & Biological Laboratories, Nagoya, Japan); monoclonal mouse anti-oligodendrocytes (clone RIP) IgG (1 : 20 000, Chemicon); and polyclonal rabbit anti-GFAP IgG (1 : 100, Sigma). After washing for 30 min (three washes of 10 min each) with PBST, brain sections were incubated in the appropriate secondary antibodies conjugated with either Cy2, Cy3 or Cy5 (1 : 200, Jackson ImmunoResearch, West Grove, PA, USA) for 4 h at 4 °C and washed with PBST for 30 min. Some of the staining sections were mounted with solution containing TO-PRO3 (1 : 1000, Molecular Probes, Eugene, OR, USA) and then examined using a confocal laser microscope (LSM510META Ver. 3.2; Carl Zeiss).

Cell counting procedure

Coronal brain sections (30 μm thickness) were randomly selected from 2.30 mm to 3.80 mm posterior to the bregma from each animal. Confocal images were captured at 1-μm intervals along the Z-axis (depth) from each section and were reconstructed into three-dimensional images. The percentages of cells expressing each cellular marker among all BrdU-labeled cells were evaluated in the neocortical parenchyma, including the motor cortex, somatosensory cortex and auditory cortex of both hemispheres of three coronal sections from each animal. The proportion of DCX(+) cells in the neocortical NG2(+) cells was estimated in 12 square areas (150 × 150 μm), which were randomly selected in the neocortical areas described above from each hemisphere. Vascular cells, including endothelial and perivascular cells, which were defined as cells having crescent or narrow nuclei located along the wall of the blood vessels, were omitted from analysis. Data from each animal were averaged.

Results

NG2-immunopositive [NG2(+)] cells were abundantly observed in all cortical layers in the adult rat neocortex (Fig. 1A). Some were closely attached to the somata of the NeuN(+) neurons (perineuronal territory, Kataoka *et al.*, 2006; arrowhead in Fig. 1B). We investigated whether neocortical cells express immature neuron markers by immunofluorescent staining for neuronal-lineage markers (PSA-NCAM and DCX). The studies revealed that many DCX(+) cells existed throughout the neocortex (Fig. 1C), although none of the cortical cells was immunopositive for PSA-NCAM within the neocortex (data not shown). The majority of DCX(+) cortical cells were found in the perineuronal territory of NeuN(+) neurons, and the multiple processes of these cells appeared to wrap around the cell bodies of the neurons (Fig. 1D), as did some of the NG2(+) cells (arrowhead in Fig. 1B). In order to investigate the coexistence of DCX and NG2 in these cells, we then performed triple immunofluorescent staining of DCX, NG2 and TO-PRO3, a DNA/RNA marker contained abundantly in neuronal somata. The study clearly revealed that such NG2(+) cells in the perineuronal territory had positive immunoreactivity for DCX (arrowheads in Fig. 2A–D), and that DCX(–)/NG2(+) cells were also observed in the cortex (arrows in Fig. 2A–D) but not in the perineuronal territory. As shown in Fig. 2G, cell pairs consisting of a DCX(+)/NG2(+) cell (arrowhead) and a DCX(–)/NG2(+) cell (arrow) were observed, suggesting that these distinctive cells originated from identical cells. Cell counting studies showed that 63 ± 3% (mean ± SD, $n = 2938$ cells from five animals) of neocortical NG2(+) cells were immunopositive for DCX. The cell densities of DCX(+)/NG2(+) cells were 70 ± 7 cells/mm^2 (mean ± SD, $n = 5$ animals) in the motor cortex (M1 and M2 defined by Paxinos & Watson, 1998), 75 ± 7 cells/mm^2 in the somatosensory cortex (S1BF and S1Tr) and 68 ± 5 cells/mm^2 in the auditory cortex (Au1, Au and AuD). These results indicate that two subtypes of NG2(+) cells exist in the neocortex, based on the presence or absence of DCX co-expression. DCX(+)/NG2(–) cells were also very occasionally observed in the adult rat neocortex (asterisk in Fig. 2H–L).

It has been reported that immature neuron markers such as PSA-NCAM and DCX are expressed only in cells observed in the entorhinal and piriform cortex (Seki & Arai, 1991; Nacher *et al.*, 2001), and that the majority of DCX-expressing cells are

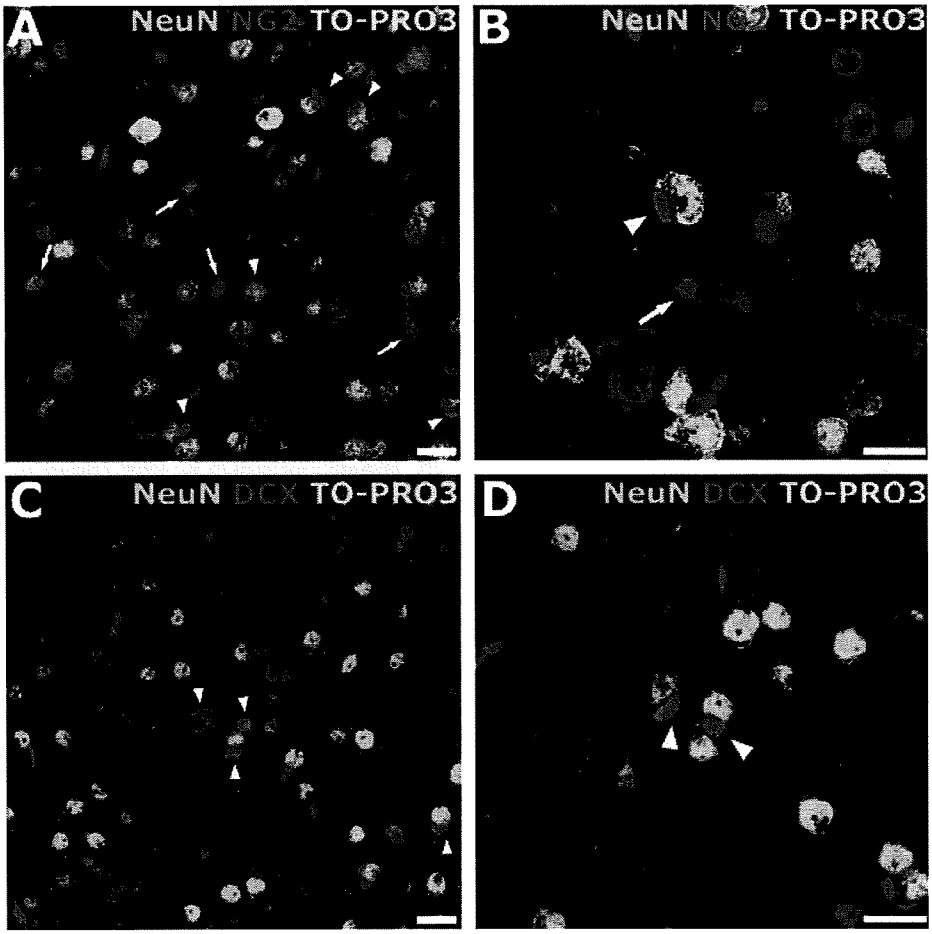

FIG. 1. The existence of NG2- or doublecortin (DCX)-positive cells in the neocortex of adult rats. (A) NG2(+) cells (red) were abundantly present in the neocortex. (B) An NG2(+) cell (arrowhead) close to NeuN(+) neuronal soma (green) and NG2(+) cell (arrow) out of the perineuronal territory. (C and D) DCX(+) cells (red) located in the perineuronal territory of NeuN(+) neurons (green). All cell nuclei and neuronal somata were stained with TO-PRO3. Scale bars: 20 μm.

immunopositive for PSA-NCAM in these areas of adult rats (Nacher *et al.*, 2001). In the present study, we found DCX(+) cells throughout all layers of the neocortex including the motor cortex, somatosensory cortex and auditory cortex, in addition to the entorhinal cortex and piriform cortex. Triple staining revealed that the cortical DCX(+)/NG2(+) cells were not immunopositive for PSA-NCAM; Fig. 3 shows that PSA-NCAM was observed only in DCX(+)/NG2(–) cells (arrows), but not in DCX(+)/NG2(+) cells (arrowheads) in the piriform cortex. These observations indicate that DCX(+)/NG2(+) cells ubiquitously observed in the adult cortex and DCX(+)/PSA-NCAM(+) cells in the piriform cortex are distinct cell populations.

NG2(+) cells have been reported to be the major population of proliferating cells in the cerebral cortex of adult rats, based on analysis of BrdU-incorporated cell nuclei 2 h after BrdU injection (Dawson *et al.*, 2003; Dayer *et al.*, 2005; Kataoka *et al.*, 2006). In the present study, we determined whether cortical proliferating cells are DCX(+)/NG2(+) cells or DCX(–)/NG2(+) cells by triple staining for NG2, DCX and BrdU using the same BrdU labeling method (Table 1). Almost all the BrdU-incorporated cells (94.2 ± 1.8%; $n = 5$ animals) were DCX(+)/NG2(+) cells (Fig. 4A–D), while none of the DCX(–)/NG2(+) cells or DCX(+)/NG2(–) cells showed BrdU incorporation at 2 h after BrdU injection (Table 1). We also confirmed that almost all the Ki67-positive cells were DCX(+)/NG2(+) cells (data not shown), indicating that these cells are the major cell population entering the cell cycle. The remaining proliferating cells were microglia showing immunoreactivity for OX-42 (data not shown).

We investigated cellular differentiation after proliferation of DCX(+)/NG2(+) cells in the adult neocortex by multiple immunodetection of cell differentiation markers and BrdU at various time points after BrdU injection. At 14 days after BrdU injection, approximately 80% (79.5 ± 3.8%), 10% (9.5 ± 3.4%) and 1% (0.61 ± 0.44%) of BrdU-labeled cells (1168 cells from five animals) were DCX(+)/NG2(+) (small arrow in Fig. 4E), DCX(–)/NG2(+) (big arrow in Fig. 4H) and DCX(+)/NG2(–) cells (arrowheads in Fig. 4E and I), respectively (Table 1). These results indicate that DCX(+)/NG2(+) cells may produce both DCX(+)/NG2(–) and DCX(–)/NG2(+) cells in addition to the reproduction of DCX(+)/NG2(+) cells. We performed other multiple immunohistochemical staining analyses of these cells to determine whether the cells were committed to the neuronal or glial cell lineage. TUC-4, a neuronal lineage marker (Seki, 2002), was found in all DCX(+)/NG2(–) cells ($n = 11$ cells at 14–28 days after BrdU injection; arrowhead in Fig. 5), but not in DCX(+)/NG2(+) or DCX(–)/NG2(+) cells, suggesting that the DCX(+)/NG2(–) cells were committed to the neuronal cell lineage after generation of the cells from DCX(+)/NG2(+) cells. We were unable to detect mature neuronal markers, including NeuN, γ-aminobutyric acid (GABA) or GAD-67, in BrdU-incorporated cells at 14 and 28 days after BrdU injection.

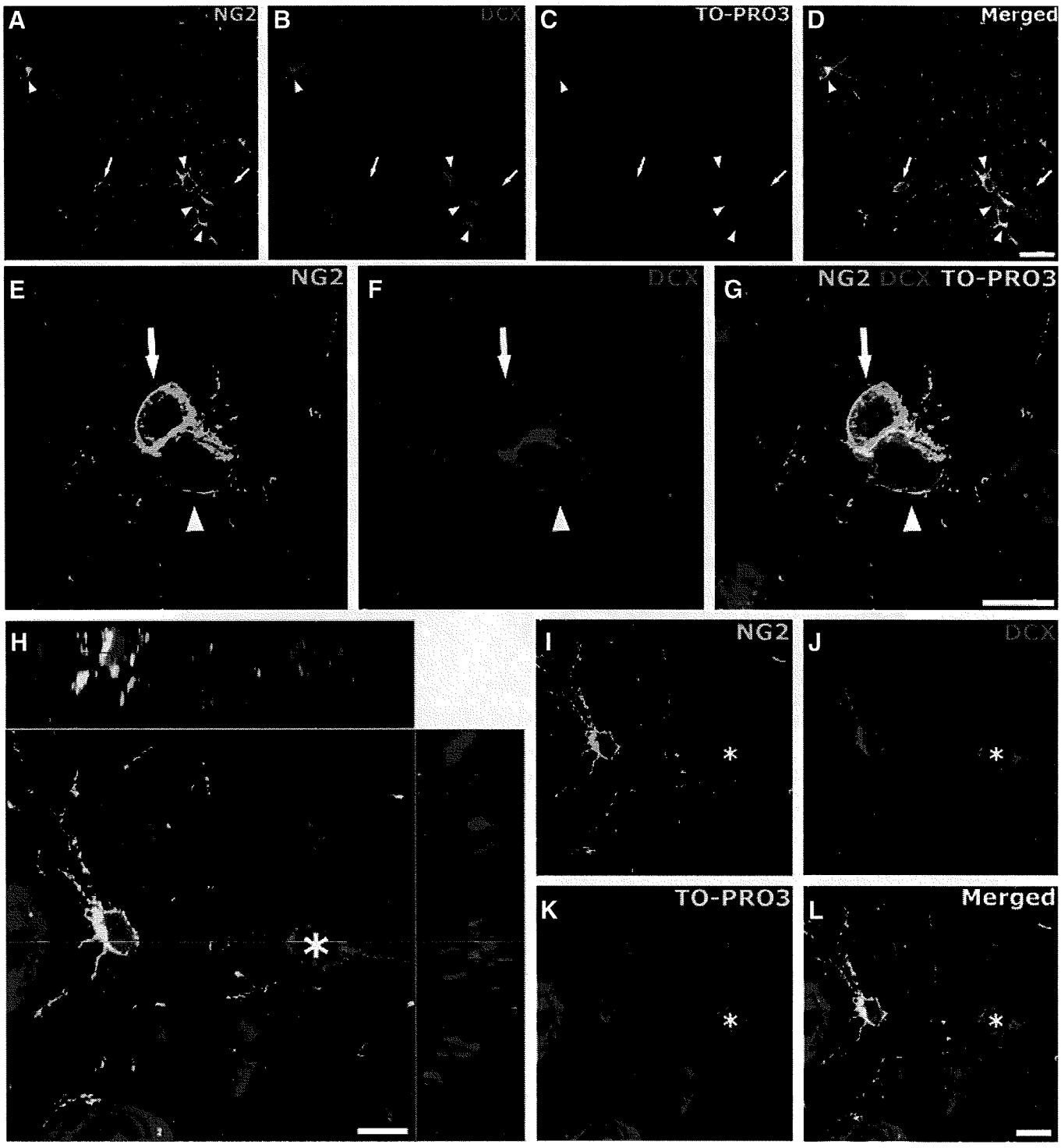

FIG. 2. Two subtypes of NG2(+) cells were distinguished with triple staining for NG2 (green), doublecortin (DCX; red) and TO-PRO3 (blue) in the neocortex. Arrowheads, DCX(+)/NG2(+) cells (yellow in D and G); arrows, DCX(–)/NG2(+) cells; asterisks, an NG2(–)/DCX(+) cell. Scale bars: 20 μm (A–D); 10 μm (E–L).

In order to examine differentiation of DCX(+)/NG2(+) cells into the glial lineage, we performed triple immunohistochemistry for BrdU, NG2 and GST-pi, an oligodendrocyte lineage marker, or for BrdU, DCX and GST-pi. At 14 days after BrdU injection, a portion of BrdU(+)/NG2(+) cells started to show faint immunoreactivity for GST-pi in the cytoplasm (Fig. 6A–D). GST-pi immunoreactivity was detected in all DCX(–)/NG2(+) cells found in this study, but not in BrdU(+)/NG2(–) and BrdU(+)/DCX(+) cells. These findings suggested that only DCX(–)/NG2(+) cells were committed to the oligodendrocyte lineage at that time point. Furthermore, among the BrdU-labeled cells at Day 28, potent GST-pi immunoreactivity was observed only in DCX(–)/NG2(–) cells (Fig. 6E–H), and was faint in DCX(–)/NG2(+) cells, but not at all present in any DCX(+) cells (Table 1). Immunohistochemical staining for RIP (Friedman *et al.*,

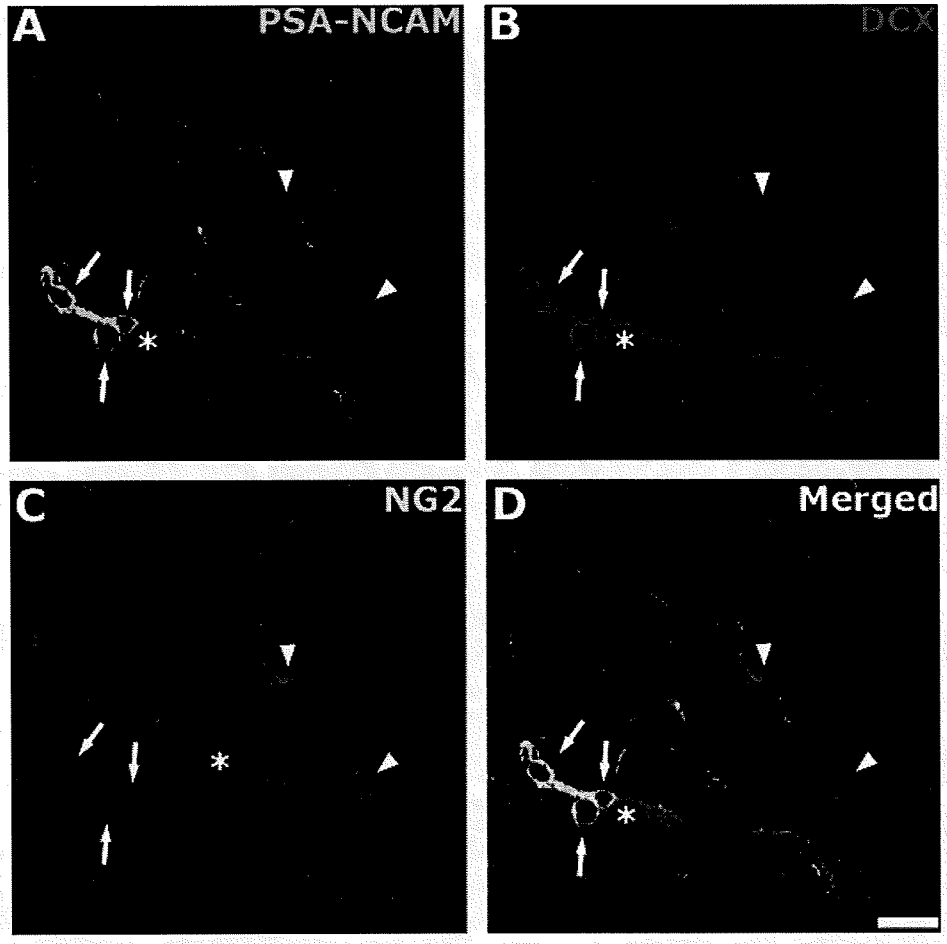

FIG. 3. Doublecortin (DCX)(+)/NG2(+) cells were a different population from DCX(+)/polysialyated form of neural cell adhesion molecule (PSA-NCAM)(+) cells in the piriform cortex. (A–D) Triple immunohistochemistry for PSA-NCAM (green), DCX (red) and NG2 (blue) in the piriform cortex. Arrowheads, DCX(+)/NG2(+) cells not immunopositive for PSA-NCAM; arrows, DCX(+)/PSA-NCAM(+) cells not immunopositive for NG2. Asterisks, DCX(+) cell not immunopositive for NG2 nor PSA-NCAM (referred to in Nacher *et al.*, 2001). Scale bar: 20 μm.

TABLE 1. Percentages of cells immunopositive for various cell differentiation markers in all BrdU-labeled cells

	BrdU-labeled cells immunopositive for each marker after injection of BrdU (%)		
	After 2 h	After 14 days	After 28 days
DCX(+)/NG2(+)	94.2 ± 1.8 (450/479)	79.5 ± 3.8 (932/1168)	69.4 ± 3.2 (584/843)
DCX(+)/NG2(–)	0 (0/479)	0.61 ± 0.44 (7/1168)	0.52 ± 0.69 (4/843)
DCX(–)/NG2(+)	0 (0/479)	9.5 ± 3.4 (112/1168)	11.0 ± 1.8 (95/843)
NG2(+)/GST-pi(+)	0 (0/213)	10.2 ± 2.6 (52/519)	12.4 ± 2.8 (48/373)
NG2(–)/GST-pi(+)	0 (0/213)	0 (0/519)	9.2 ± 1.2 (35/373)

Data are presented as mean ± SEM (%), $n = 5$ animals at each time point. The number of positive cells for each marker/number of BrdU-labeled cells is given in parentheses. DCX, doublecortin; GST, glutathione S-transferase.

1989), another mature oligodendrocyte marker, revealed that this immunoreactivity was detected in most of the DCX(–)/NG2(–)/GST-pi(+) cells (Fig. 6I–K).

Discussion

In the present study, we have provided novel evidence that the adult rat neocortex ubiquitously possesses DCX(+)/NG2(+) cells and that these cells are the major population of cortical cells involved in cell reproduction. The DCX(+)/NG2(+) cells differentiate into two kinds of cell lineages [DCX(+)/NG2(–) cells and DCX(–)/NG2(+) cells] at 14–28 days after mitosis, as well as undergoing reproduction into identical cells. DCX(+)/NG2(–) cells were immunoreactive for TUC-4, a neuronal lineage marker, whereas DCX(–)/NG2(+) cells started to co-express an oligodendrocyte marker GST-pi. Furthermore, the NG2(+)/GST-pi(+) cells differentiated into new mature oligodendrocytes at 28 days. These results indicate that the DCX(+)/NG2(+) cells serve as cortical progenitor cells, retaining the potential to produce neuronal lineage cells and oligodendrocytes in the neocortex (Fig. 7).

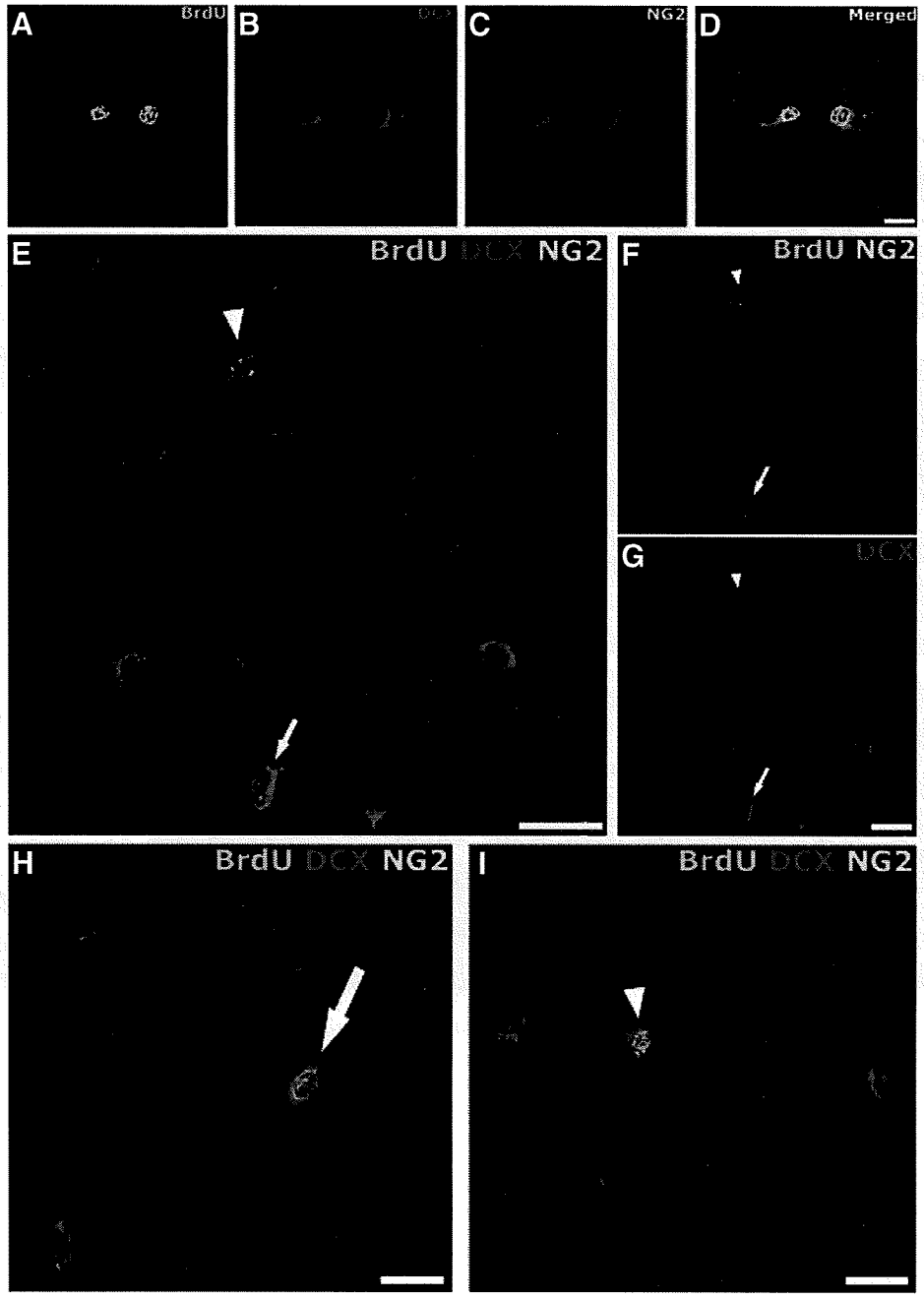

FIG. 4. (A–D) 5′-Bromodeoxyuridine (BrdU)-incorporated nuclei (green) in NG2(+)/doublecortin (DCX)(+) cells in the neocortex at 2 h after BrdU injection: (red), DCX; (blue), NG2. (E–I) Multi-differentiation of BrdU-labeled NG2(+)/DCX(+) cells in the neocortex at 14 days after BrdU injection: BrdU (green), DCX (red) and NG2 (blue). Arrowheads in (E–G), BrdU-labeled DCX(+)/NG2(–) cells; (E–G) indicate the same area. Small arrows in (E–G), a BrdU-labeled DCX(+)/NG2(+) cell. Big arrow in (H), a BrdU-labeled DCX(–)/NG2(+) cell. Scale bars: 10 μm (A–D); 20 μm (E–I).

Two subtypes of NG2-positive cells in the neocortex of adult rats

It has been known that NG2(+) cells abundantly and broadly exist in the cerebral cortex of adult rats. We observed that 1.2 ± 0.4% and 1.8 ± 0.7% of NG2(+) cells (813 cells and 946 cells from five animals) in the neocortex were immunoreactive for BrdU and Ki67, respectively, 2 h after BrdU injection. The findings suggested that few cortical NG2(+) cells are involved in the cell cycle, and are supported by a previous report that only 1.5% of all NG2-positive cells are labeled with BrdU 2 h after BrdU injection (Dawson *et al.*, 2003). In the spinal cord of adult rats, two subtypes of NG2-positive cells were argued based on their response to experimental demyelination (Keirstead *et al.*, 1998). Thus, we assumed that two distinct populations of NG2(+) cells were also present in the adult cortex, and confirmed this finding based on co-expression of DCX (Fig. 2). Proliferative activity was observed only in the DCX(+)/NG2(+) cells, but not in the DCX(–)/NG2(+) cells (Fig. 4D). Furthermore, we confirmed that none of the DCX(+)/NG2(+) cells in the neocortex was immunopositive for GFAP or nestin. Our previous studies showed that the majority of proliferating cells are located close to the neuronal somata (the perineuronal territory) in the cerebral cortex of adult rats (Kataoka *et al.*, 2006). Indeed, in the present study, most of the

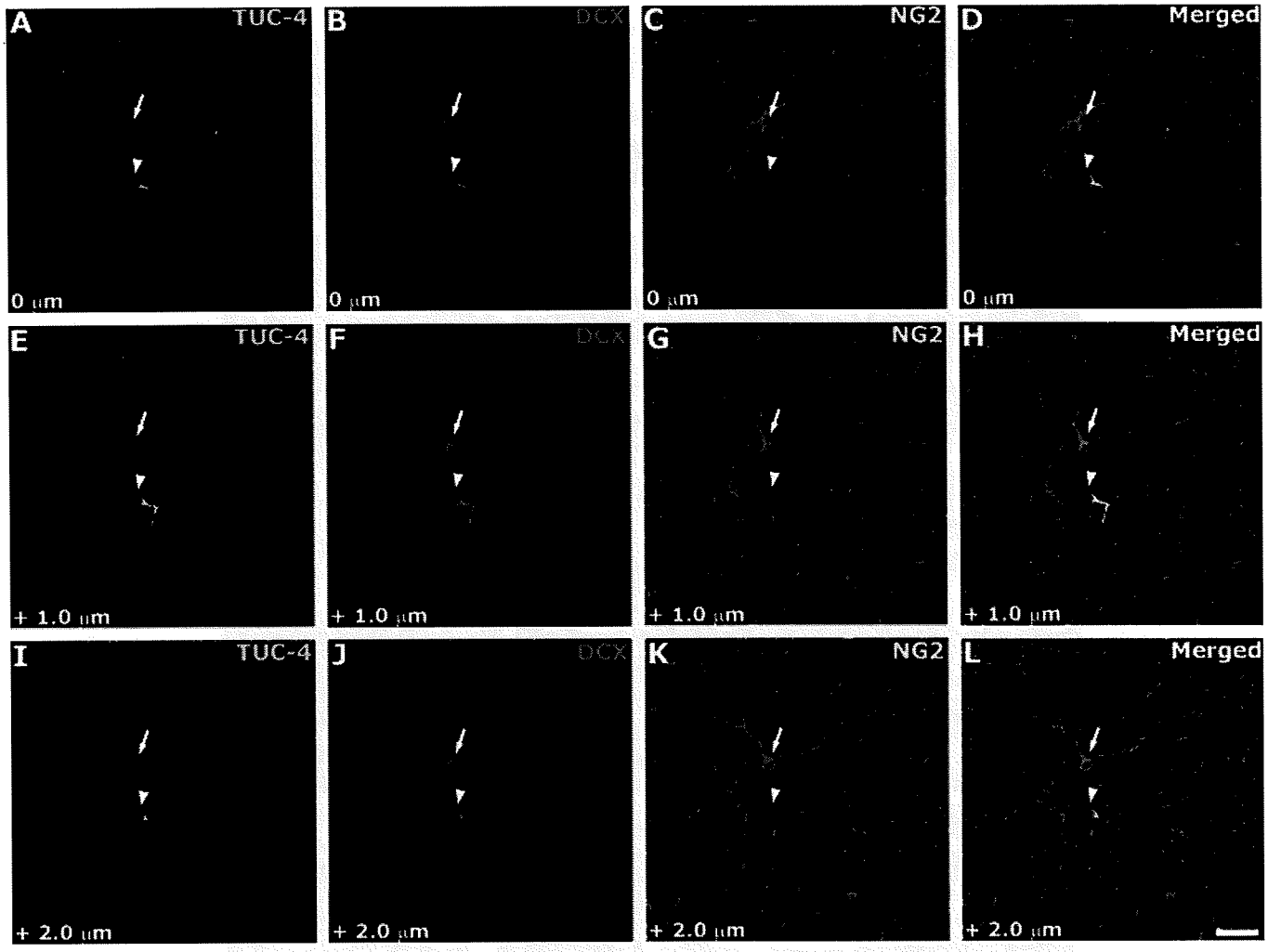

FIG. 5. A neuronal lineage marker (TUC-4)-expressing doublecortin (DCX)(+)/NG2(–) cells. Sequential confocal images (0 to +2 μm; A–D, 0μm; E–H, +1.0μm; I–L, +2.0μm) captured at 1-μm intervals for TUC-4 (green), DCX (red) and NG2 (blue). Arrowheads, DCX(+)/NG2(–) cells immunopositive for TUC-4; arrows, DCX(+)/NG2(+) cells not immunopositive for TUC-4. Scale bar: 20 μm.

DCX(+)/NG2(+) cells were observed in the perineuronal territory, and many DCX(–)/NG2(+) cells were outside this area (data not shown). These findings suggest that the DCX(+)/NG2(+) cells are 'cortical progenitor cells' in the neocortex.

Existence of DCX(+) cells in the adult neocortex

In the present study, we observed that the DCX(+)/NG2(+) cells exist throughout the neocortex of adult rats under physiological conditions (Fig. 2). However, previous studies in adult rats have reported that DCX(+) cells are observed only in cortical layers II or III of the piriform cortex, and in layer II of the entorhinal, perirhinal and insular cortex (Nacher *et al.*, 2001, 2004), but not in the neocortex more dorsal to these regions (Jin *et al.*, 2003; Yang *et al.*, 2004; Dayer *et al.*, 2005). Indeed, the intensity of DCX immunoreactivity in the DCX(+)/NG2(+) cells observed in the present study was weaker than that in the previously reported DCX(+) cells in the piriform cortex (Fig. 3D). Thus, we could barely immunodetect DCX in the DCX(+)/NG2(+) cells at lower magnifications using a confocal laser-scanning microscope; however, we were able to successfully detect them at higher magnifications, i.e. ×40 or ×63 at the objective lens. Furthermore, we confirmed the expression of DCX in the neocortex using Western blot analysis; a single 40-kDa band of DCX protein (Brown *et al.*, 2003) was detected in tissue homogenates prepared from the parietal cortical tissue as well as piriform cortex (data not shown).

Cellular differentiation of the DCX(+)/NG2(+) cells

In the current study, we showed that DCX(+)/NG2(–) cells are generated from DCX(+)/NG2(+) cells in the neocortex of adult rats using BrdU-labeling methods (Fig. 4 and Table 1), and that all the DCX(+)/NG2(–) cells contain TUC-4 (Fig. 5). In the dentate gyrus of the hippocampus, it has been shown that a subset of NG2(+) cells can differentiate into GABAergic neurons, upregulating the expression of TOAD-64 (referred to as TUC-4) and downregulating the expression of NG2 (Belachew *et al.*, 2003). We confirmed that the NG2(+) cells in the dentate gyrus were also immunoreactive for DCX (data not shown). Furthermore, DCX-expressing NG2(+) progenitor cells are also observed in the anterior SVZ, and these cells differentiate into GABAergic interneurons in the olfactory bulb. Such NG2(+) cells of these neurogenic regions differentiate into GABAergic interneurons following downregulation of the NG2 expression (Aguirre & Gallo, 2004). The possibility exists that the DCX(+)/NG2(+) cells in the

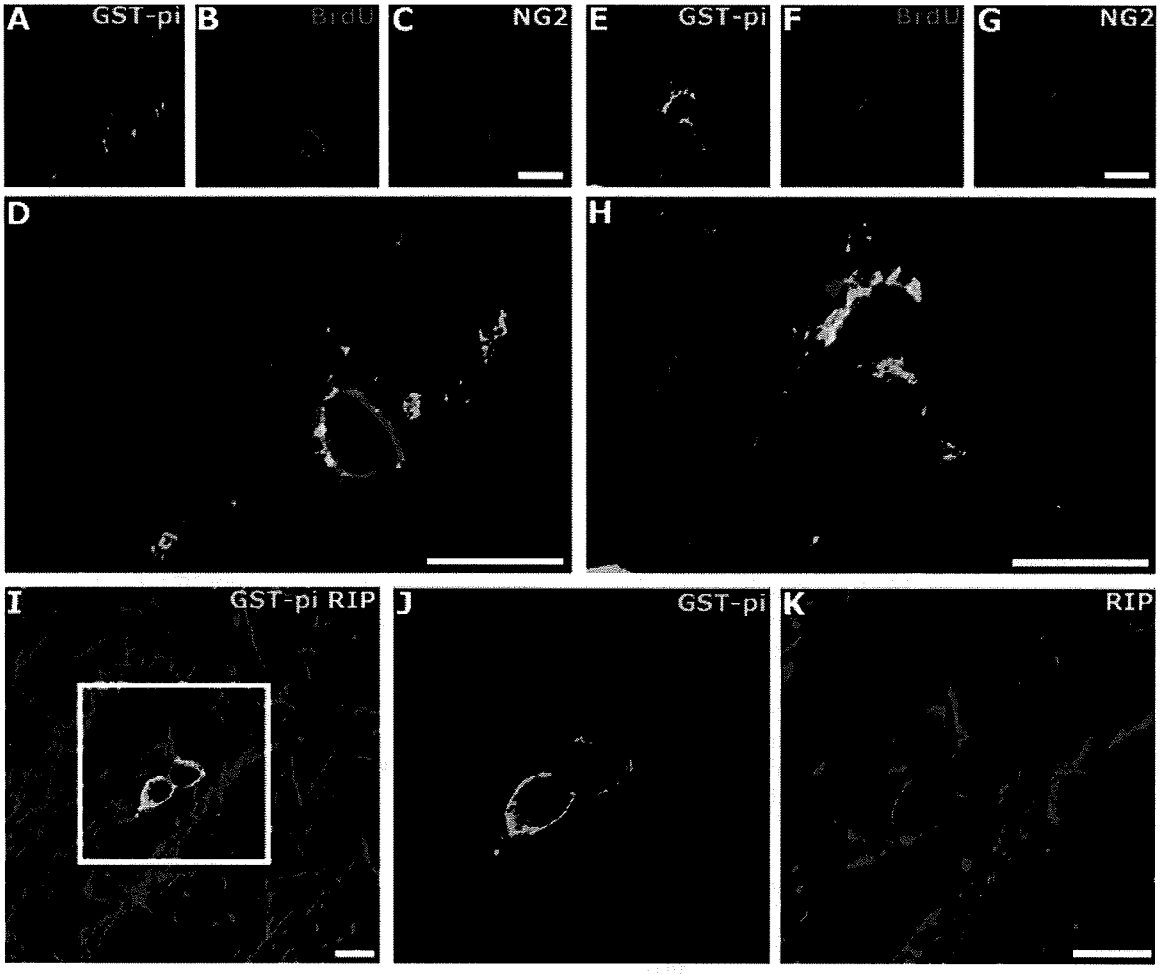

FIG. 6. Glial lineage markers expressing DCX(–)/NG2(+) cells. (A–D) A 5′-bromodeoxyuridine (BrdU)-labeled NG2(+)/glutathione S-transferase (GST)-pi(+) cell in the neocortex at 14 days after BrdU injection. (E–H) A BrdU-labeled NG2(–)/GST-pi(+) cell in the neocortex at 28 days after BrdU injection: GST-pi (green), BrdU (red) and NG2 (blue). (I–K) Two GST-pi(+)/RIP(+) cells in the neocortex, GST-pi (green) and RIP (blue). (G and K) Magnified views of the area indicated by a white square in (I). Scale bars: 10 μm.

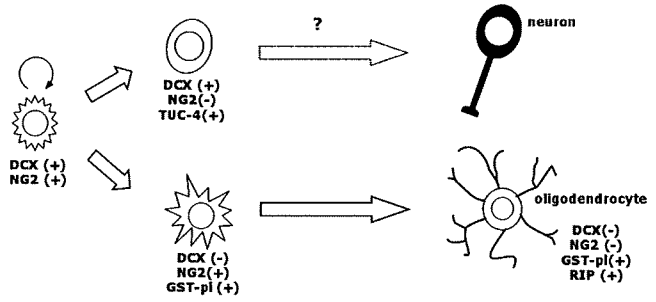

FIG. 7. Schematic representation of a hypothetical differentiation model of doublecortin (DCX)(+)/NG2(+) cells in the adult neocortex. DCX(+)/NG2(+) cells reproduce the identical cells, DCX(+)/NG2(–) cells and DCX(+)/NG2(–) cells. In the neuronal lineage, newly generated DCX(+)/NG2(–) cells show TUC-4 immunoreactivity. In the oligodendrocyte differentiation, newly generated DCX(–)/NG2(+) cells show glutathione S-transferase (GST)-pi immunoreactivity, and the cells further differentiate into mature oligodendrocytes immunopositive for GST-pi and RIP. During oligodendrocyte differentiation, the expression of NG2 is downregulated, whereas that of cytoplasmic GST-pi is upregulated.

adult neocortex also differentiate into GABAergic interneurons with downregulation of NG2 expression. In the present study, we were able to demonstrate that neocortical DCX(+)/NG2(+) cells have the potential to give rise to DCX(+)/NG2(–) cells committed to neuronal lineage cells, but found that none of the cells indicated immunohistochemical characteristics of mature GABAergic neurons. Under such a low-stimulative environment as in the present experiment, rodent neocortex might not need to generate new neurons. In fact, it has been reported that neurogenesis occurs in the cerebral cortex of adult mice under pathological conditions (Magavi *et al.*, 2000). Furthermore, in the present study we were unable to determine the fates and functions of newly generated DCX(+)/NG2(–) cells in the neocortex. Although those cells were not immunohistochemically mature neurons, such neuronal lineage cells might have physiological functions even at immature stages. A subpopulation of immature cells in the cortex has been reported to show physiological properties, including ion channel expression profiles and depolarization-induced multiple spikes (Chittajallu *et al.*, 2004).

At 2 h after BrdU injection, a small number of dividing cells were OX-42(+) microglia. Thus, we cannot rule out the possibility that the DCX(+)/NG2(–) cells were generated from the microglia *in situ* in the neocortex. However, until now microglia has not been reported to give rise to neurons or other glial cells, including astrocytes, oligodendrocytes and NG2(+) glial progenitor cells in the adult brain under physiological conditions. Indeed, we observed that none of the OX-42(+) cells was immunoreactive for NG2 and/or DCX in the

neocortex. The origin of the newly generated DCX(+)/NG2(–) cells may be related to BrdU(+)/DCX(+) cells originating in the SVZ migrating into the neocortex under pathological conditions, as shown by Magavi *et al.* (2000). In the neonatal brain, newly generated DCX(+) cells have also been reported to migrate from the SVZ to the cerebral cortex (Suzuki & Goldman, 2003; Fagel *et al.*, 2006). The vast majority of the DCX(+) young neurons migrating from the SVZ have been known to express PSA-NCAM (Doetsch *et al.*, 1997, 1999; Fagel *et al.*, 2006). However, the newly generated DCX(+)/NG2(–) cells observed in the present study were not immunopositive for PSA-NCAM (data not shown). Based on these findings we suggest that the DCX(+)/NG2(–) cells were likely derived from the DCX(+)/NG2(+) cells *in situ* in the neocortical parenchyma.

Dawson *et al.* (2003) suggested that cortical NG2(+) cells can generate new oligodendrocytes, by finding BrdU-labeled cells expressing 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase), an oligodendrocyte marker. However, that study did not trace the differentiation between NG2-expressing cells and CNPase-expressing mature oligodendrocytes, and could not show that NG2(+) cells were on the identical cell lineage path of mature oligodendrocytes. In the present study, using GST-pi, an oligodendrocyte lineage marker, we were able to trace every stage of oligodendrocyte differentiation by NG2(+)/GST-pi(+) cells from DCX(+)/NG2(+) progenitor cells to NG2(–)/GST-pi(+) mature oligodendrocytes (Fig. 6A–H and Table 1). The NG2(–)/GST-pi(+) cells were confirmed to be mature oligodendrocytes by observation of immunoreactivity for RIP, a mature oligodendrocyte marker (Fig. 6I). These results are in agreement with previous reports indicating maturation of oligodendrocytes following the downregulation of NG2 expression (Nishiyama *et al.*, 1996) and the progressive expression of RIP (Friedman *et al.*, 1989). We have provided the first evidence that cortical NG2(+) cells co-expressing a neuronal marker reproduce mature oligodendrocytes.

It has been reported that NG2(+) cells may give rise to GFAP(+) astrocytes in the cerebral cortex of adult rats (Dawson *et al.*, 2003). In the present study, BrdU-labeled GFAP(+) astrocytes were infrequently found in the adult neocortex at 28 days after BrdU injection (less than 1%, 2/456 cells from five animals). Thus, perineuronal DCX(+)/NG2(+) cells might differentiate into astrocytes as well as oligodendrocytes and neurons. We previously demonstrated that neural excitation stimulates cellular proliferation of the perineuronal cells (Tamura *et al.*, 2004; Kataoka *et al.*, 2006), and such an excitation facilitates the production of GFAP(+) astrocytes (unpublished observation). These observations suggest that local neural activity controls the production rates of astrocytes, oligodendrocytes and neurons by regulating the proliferation rate and the direction of the differentiation of the DCX(+)/NG2(+) cells.

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Abbreviations

BrdU, 5′-bromodeoxyuridine; CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; DCX, doublecortin; GABA, γ-aminobutyric acid; GFAP, glial fibrillary acidic protein; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PBST, 0.3% Triton X-100 in PBS; PSA-NCAM, polysialyated form of neural cell adhesion molecule; SVZ, subventricular zone.

References

Aguirre, A. & Gallo, V. (2004) Postnatal neurogenesis and gliogenesis in the olfactory bulb from NG2-expressing progenitors of the subventricular zone. *J. Neurosci.*, **24**, 10530–10541.

Belachew, S., Chittajallu, R., Aguirre, A.A., Yuan, X., Kirby, M., Anderson, S. & Gallo, V. (2003) Postnatal NG2 proteoglycan-expressing progenitor cells are intrinsically multipotent and generate functional neurons. *J. Cell Biol.*, **161**, 169–186.

Brown, J.P., Couillard-Despres, S., Cooper-Kuhn, C.M., Winkler, J., Aigner, L. & Kuhn, H.G. (2003) Transient expression of doublecortin during adult neurogenesis. *J. Comp. Neurol.*, **467**, 1–10.

Cameron, H.A., Woolley, C.S., McEwen, B.S. & Gould, E. (1993) Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. *Neuroscience*, **56**, 337–344.

Chittajallu, R., Aguirre, A. & Gallo, V. (2004) NG2-positive cells in the mouse white and grey matter display distinct physiological properties. *J. Physiol.*, **561**, 109–122.

Dawson, M.R., Polito, A., Levine, J.M. & Reynolds, R. (2003) NG2-expressing glial progenitor cells: an abundant and widespread population of cycling cells in the adult rat CNS. *Mol. Cell. Neurosci.*, **24**, 476–488.

Dayer, A.G., Cleaver, K.M., Abouantoun, T. & Cameron, H.A. (2005) New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. *J. Cell Biol.*, **168**, 415–427.

Doetsch, F., Caille, I., Lim, D.A., Garcia-Verdugo, J.M. & Alvarez-Buylla, A. (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell*, **97**, 703–716.

Doetsch, F., Garcia-Verdugo, J.M. & Alvarez-Buylla, A. (1997) Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J. Neurosci.*, **17**, 5046–5061.

Doetsch, F., Petreanu, L., Caille, I., Garcia-Verdugo, J.M. & Alvarez-Buylla, A. (2002) EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. *Neuron*, **36**, 1021–1034.

Fagel, D.M., Ganat, Y., Silbereis, J., Ebbitt, T., Stewart, W., Zhang, H., Ment, L.R. & Vaccarino, F.M. (2006) Cortical neurogenesis enhanced by chronic perinatal hypoxia. *Exp. Neurol.*, **199**, 77–91.

Friedman, B., Hockfield, S., Black, J.A., Woodruff, K.A. & Waxman, S.G. (1989) In situ demonstration of mature oligodendrocytes and their processes: an immunocytochemical study with a new monoclonal antibody, rip. *Glia*, **2**, 380–390.

Gage, F.H., Coates, P.W., Palmer, T.D., Kuhn, H.G., Fisher, L.J., Suhonen, J.O., Peterson, D.A., Suhr, S.T. & Ray, J. (1995) Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc. Natl Acad. Sci. USA*, **92**, 11879–11883.

Gritti, A., Frolichsthal-Schoeller, P., Galli, R., Parati, E.A., Cova, L., Pagano, S.F., Bjornson, C.R. & Vescovi, A.L. (1999) Epidermal and fibroblast growth factors behave as mitogenic regulators for a single multipotent stem cell-like population from the subventricular region of the adult mouse forebrain. *J. Neurosci.*, **19**, 3287–3297.

Jin, K., Sun, Y., Xie, L., Peel, A., Mao, X.O., Batteur, S. & Greenberg, D.A. (2003) Directed migration of neuronal precursors into the ischemic cerebral cortex and striatum. *Mol. Cell. Neurosci.*, **24**, 171–189.

Kataoka, Y., Tamura, Y., Takamori, Y., Cui, Y. & Yamada, H. (2006) Perineuronal germinal cells in the rat cerebral cortex. *Med. Mol. Morph.*, **39**, 28–32.

Keirstead, H.S., Levine, J.M. & Blakemore, W.F. (1998) Response of the oligodendrocyte progenitor cell population (defined by NG2 labelling) to demyelination of the adult spinal cord. *Glia*, **22**, 161–170.

Lois, C. & Alvarez-Buylla, A. (1993) Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. *Proc. Natl Acad. Sci. USA*, **90**, 2074–2077.

Magavi, S.S., Leavitt, B.R. & Macklis, J.D. (2000) Induction of neurogenesis in the neocortex of adult mice. *Nature*, **405**, 951–955.

Morshead, C.M., Reynolds, B.A., Craig, C.G., McBurney, M.W., Staines, W.A., Morassutti, D., Weiss, S. & van der Kooy, D. (1994) Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. *Neuron*, **13**, 1071–1082.

Nacher, J., Crespo, C. & McEwen, B.S. (2001) Doublecortin expression in the adult rat telencephalon. *Eur. J. Neurosci.*, **14**, 629–644.

Nacher, J., Pham, K., Gil-Fernandez, V. & McEwen, B.S. (2004) Chronic restraint stress and chronic corticosterone treatment modulate differentially the expression of molecules related to structural plasticity in the adult rat piriform cortex. *Neuroscience*, **126**, 503–509.

Nishiyama, A., Chang, A. & Trapp, B.D. (1999) NG2+ glial cells: a novel glial cell population in the adult brain. *J. Neuropathol. Exp. Neurol.*, **58**, 1113–1124.

Nishiyama, A., Lin, X.H., Giese, N., Heldin, C.H. & Stallcup, W.B. (1996) Interaction between NG2 proteoglycan and PDGF alpha-receptor on O2A progenitor cells is required for optimal response to PDGF. *J. Neurosci. Res.*, **43**, 315–330.

Palmer, T.D., Markakis, E.A., Willhoite, A.R., Safar, F. & Gage, F.H. (1999) Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J. Neurosci.*, **19**, 8487–8497.

Paxinos, G. & Watson, C. (1998) *The Rat Brain in Stereotaxic Coordinates*, 4th Edn. Academic Press, San Diego.

Seki, T. (2002) Expression patterns of immature neuronal markers PSA-NCAM, CRMP-4 and NeuroD in the hippocampus of young adult and aged rodents. *J. Neurosci. Res.*, **70**, 327–334.

Seki, T. & Arai, Y. (1991) Expression of highly polysialylated NCAM in the neocortex and piriform cortex of the developing and the adult rat. *Anat. Embryol. (Berl.)*, **184**, 395–401.

Seri, B., Garcia-Verdugo, J.M., McEwen, B.S. & Alvarez-Buylla, A. (2001) Astrocytes give rise to new neurons in the adult mammalian hippocampus. *J. Neurosci.*, **21**, 7153–7160.

Suzuki, S.O. & Goldman, J.E. (2003) Multiple cell populations in the early postnatal subventricular zone take distinct migratory pathways: a dynamic study of glial and neuronal progenitor migration. *J. Neurosci.*, **23**, 4240–4250.

Tamura, Y., Kataoka, Y., Cui, Y. & Yamada, H. (2004) Cellular proliferation in the cerebral cortex following neural excitation in rats. *Neurosci. Res.*, **50**, 129–133.

Yang, H.K., Sundholm-Peters, N.L., Goings, G.E., Walker, A.S., Hyland, K. & Szele, F.G. (2004) Distribution of doublecortin expressing cells near the lateral ventricles in the adult mouse brain. *J. Neurosci. Res.*, **76**, 282–295.

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INTRACELLULAR TRANSLOCATION OF GLUTATHIONE S-TRANSFERASE PI DURING OLIGODENDROCYTE DIFFERENTIATION IN ADULT RAT CEREBRAL CORTEX *IN VIVO*

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Abstract—Glutathione S-transferase (GST)-pi is a cytosolic isoenzyme used as a marker for mature oligodendrocytes in the mammalian brain. However, the cellular properties of GST-pi-immunoreactive [GST-pi (+)] cells in adult brain are not completely understood. We immunohistochemically demonstrated the existence of two subtypes of GST-pi (+) cells in the cerebral cortex of adult rats: one subtype exhibited GST-pi in the cytoplasm (C-type cells), while the other did mainly in the nucleus (N-type cells). The GST-pi (+) C-type cells were also immunopositive for 2′,3′-cyclic nucleotide 3′-phosphodiesterase and RIP, indicating that they were mature oligodendrocytes, while the GST-pi (+) N-type cells expressed NG2, indicating that they were oligodendrocyte progenitor cells. Furthermore, observation of the fate of newly-generated cells by 5-bromodeoxyuridine-labeling revealed that the GST-pi (+) N-type cells differentiated into C-type cells. These findings indicate translocation of GST-pi from the nucleus to the cytoplasm during oligodendrocyte maturation.

Key words: oligodendrocyte progenitor cells, NG2, CNPase, RIP, BrdU.

Glutathione S-transferases (GSTs) are phase II detoxification enzymes that catalyze the conjugation of various xenobiotic and endogenous electrophiles with reduced glutathione. The mammalian GSTs consist of eight distinct classes (alpha, mu, pi, theta, sigma, kappa, zeta, and omega) separable on the basis of their biological properties (Mannervik et al., 1985; Meyer et al., 1991; Meyer and Thomas, 1995; Pemble et al., 1996; Board et al., 1997, 2000). The GST isoenzymes are widely expressed and distributed among several types of tissue including the brain (Theodore et al., 1985; Li et al., 1986; Abramovitz and Listowsky, 1987). Three GST isoenzymes (alpha, mu, and pi) have been observed in neurons and glial cells in the brain: GST-alpha is expressed in the nuclei of neurons of the rat brain (Johnson et al., 1993); GST-mu is expressed in glial fibrillary acidic protein (GFAP)–immunopositive astrocytes, but in neither neurons nor oligodendrocytes in rat brain (Abramovitz et al., 1988; Cammer and Zhang, 1992); and GST-pi is found in the cytoplasm of mature oligodendrocytes, which are immunopositive for 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase), in rodent cerebral cortex (Cammer et al., 1989; Tansey and Cammer, 1991). Of these three GSTs, GST-pi has been used as a specific marker protein for mature oligodendrocytes in the mammalian adult brain (Tanaka et al., 2003; Mason et al., 2004; Gotts and Chesselet, 2005). However, it was recently reported that glial cells containing GST-pi protein in both the cytoplasm and nucleus were observed in the gray and white matter of normal human brain (Terrier et al., 1990). Thus, the properties of these GST-pi (+) cells have yet to be determined.

In the present study, we investigated the properties of GST-pi (+) C-type cells and GST (+) N-type cells using several specific markers of the oligodendrocyte lineage, including differentiation state-specific markers. Furthermore, cell differentiation processes in this lineage were observed using a flash labeling method with 5-bromodeoxyuridine (BrdU).

EXPERIMENTAL PROCEDURES

Animals and BrdU injections

Adult male Wistar rats (SLC, Hamamatsu, Japan; 250–350 g body weight) were used. All experimental protocols were approved by the Ethics Committee on Animal Care and Use of Kansai Medical University and were performed in accordance with the Principles of Laboratory Animal Care (NIH Publication No. 85–23, revised 1985). Every effort was made to minimize the number of animals used and their suffering. In order to label proliferating cells, rats (n=9) were intraperitoneally injected with BrdU diluted with saline (50 mg/kg body weight).

Histochemistry

Animals were deeply anesthetized with diethyl ether and perfused transcardially with 4% formaldehyde buffered with 0.1 M phosphate-buffered saline (PBS; pH 7.4) 2 h, 14 days, and 28 days after BrdU injection. The brains were removed, post-fixed in 4% formaldehyde buffered with 0.1 M PBS overnight at 4 °C, and then immersed in 20% (w/v) sucrose solution. Coronal brain sections

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Abbreviations: BrdU, 5-bromodeoxyuridine; CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PBST, 0.3% Triton X-100 in phosphate-buffered saline.

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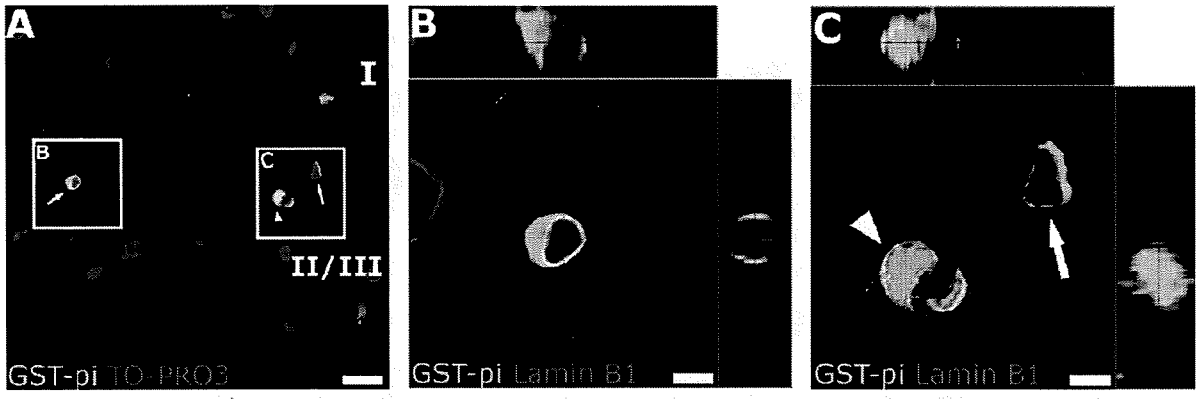

Fig. 1. Two subtypes of GST-pi (+) cells in cerebral cortex. (A) Double staining for GST-pi (green) and TO-PRO3 (red) in cortical layers I and II–III of somatosensory cortex reveals two subtypes of GST-pi (+) cells; arrows indicate cytoplasmic GST-pi (+) cells, while the arrowhead indicates a nuclear GST-pi (+) cell on the image of a single z-slice. (B, C) Single-z-slice images with ortho-images of confocal z-stacks (upper and right panels in B and C) of GST-pi (+) cells in the areas indicated by boxes in A. Double-immunostaining for GST-pi (green) and lamin B1 (red) reveals that GST-pi (+) cells include both GST-pi (+) C-type cells, which exhibit immunoreactivity for GST-pi in the cytoplasm (B, and arrow in C), and a GST-pi (+) N-type cell, which exhibits immunoreactivity mainly within the nucleus (arrowhead in C). Scale bars=20 μm in (A); 5 μm (B, C).

(30-μm thickness, 0.26 mm to 3.80 mm posterior to bregma) were prepared using a cryostat and collected as free-floating sections.

For detection of BrdU incorporation, brain sections were pre-incubated in 50% formamide/2× saline–sodium citrate buffer for 2 h at 65 °C, incubated in 2 N HCl for 30 min at 37 °C, rinsed in 0.1 M boric acid (pH 8.5) for 10 min at 25 °C, and then washed with 0.3% Triton X-100 in phosphate-buffered saline (PBST), as described previously (Tamura et al., 2004). For multiplex-immunostaining, brain sections were incubated with several primary antibodies at 4 °C for 12–36 h. The primary antibodies used in this study were as follows: monoclonal mouse anti-GST-pi IgG (1:1000, BD Biosciences, San Jose, CA, USA), polyclonal rabbit anti-GST-pi IgG (1:500, Medical and Biological Laboratories, Nagoya, Japan), monoclonal rat anti-BrdU IgG (1:10, Oxford Biotechnology, Oxford, UK), polyclonal rabbit anti-Ki67 IgG (1:1000, NovoCastra, Newcastle, UK), monoclonal mouse anti-NG2 IgG (1:200, Chemicon, Temecula, CA, USA), polyclonal rabbit anti-NG2 IgG (1:200, Chemicon), monoclonal mouse anti-CNPase IgG (1:20,000, Sigma, Taufkirchen, Germany), monoclonal mouse anti-oligodendrocytes (clone RIP) IgG (1:20,000, Chemicon), and polyclonal goat anti-lamin B1 IgG (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with PBST for 30 min, brain sections were incubated with secondary antibodies conjugated with Cy2, Cy3, or Cy5 (1:200, Jackson ImmunoResearch, West Grove, PA, USA) for 4 h at 4 °C and washed with PBST for 30 min. The sections were mounted with TO-PRO3-containing solution (Molecular Probes, Eugene, OR, USA) and examined using a confocal laser microscope (LSM510META Ver. 3.2; Carl Zeiss, Oberkochen, Germany).

Cell counting procedure

Two brain sections were randomly selected from each animal. The number of cells expressing each oligodendrocyte lineage marker among GST-pi (+) cells was counted in 72 square areas (150 μm×150 μm, 12 square areas in each hemisphere) randomly positioned in layers I–VI of cerebral cortex (motor cortex, M1 and M2; somatosensory cortex, S1BF, S1DZ, S1FL, S1HL, and S1Tr; and auditory cortex, Au1, AuD, and AuV; as defined by Paxinos and Watson, 1998). The number of cells expressing each cellular marker among all BrdU-labeled cells was counted in the same area of each animal. Data from each animal were averaged.

RESULTS

Two subtypes of GST-pi (+) cells

GST-pi (+) cells were widely present throughout layers I–VI of cerebral cortex of adult rats. Double staining for GST-pi and TO-PRO3, a DNA marker, which allows visualization of all cells, especially the nuclei, revealed two different types of GST-pi (+) cells on the basis of intracellular distribution of GST-pi immunoreactivity: cells exhibiting GST-pi immunoreactivity in the cytoplasm (C-type cells, arrows in Fig. 1A) and those exhibiting immunoreactivity in the nucleus (N-type cells, arrowhead in Fig. 1A). In order to visualize the nuclear membrane, immunohistochemical staining for lamin B1, a nuclear envelope protein (Kataoka et al., 2006; Takamori et al., 2007), was performed. Confocal z-series analysis of double-immunofluorescence staining for GST-pi and lamin B1 clearly showed that the C-type cells contained GST-pi mainly in the cytoplasm and little within the nucleus (Fig. 1B and arrow in Fig. 1C), while N-type cells contained this protein mainly in the nucleus and in small amounts in the cytoplasm, especially around the nucleus (arrowhead in Fig. 1C).

Expression of GST-pi in oligodendrocyte-lineage cells

To examine the features of GST-pi (+) C-type and N-type cells in the cerebral cortex, we performed double-immunofluorescence staining using mature oligodendrocyte cell markers (CNPase and RIP). All the cells immunopositive for CNPase were GST-pi (+) cells (arrows in Fig. 2A). However, GST-pi (+) cells without CNPase were also observed (arrowheads in Fig. 2A). Cell counting revealed that 72.6±2.8% (mean±S.D., n=1389 cells from three animals) and 72.4±1.5% (mean±S.D., n=1338 cells from three animals) of GST-pi (+) cells were CNPase (+) and RIP (+), respectively. The distribution of CNPase (+) cells was very similar to that of RIP (+) cells. The antigen recognized with the RIP antibody has recently been iden-

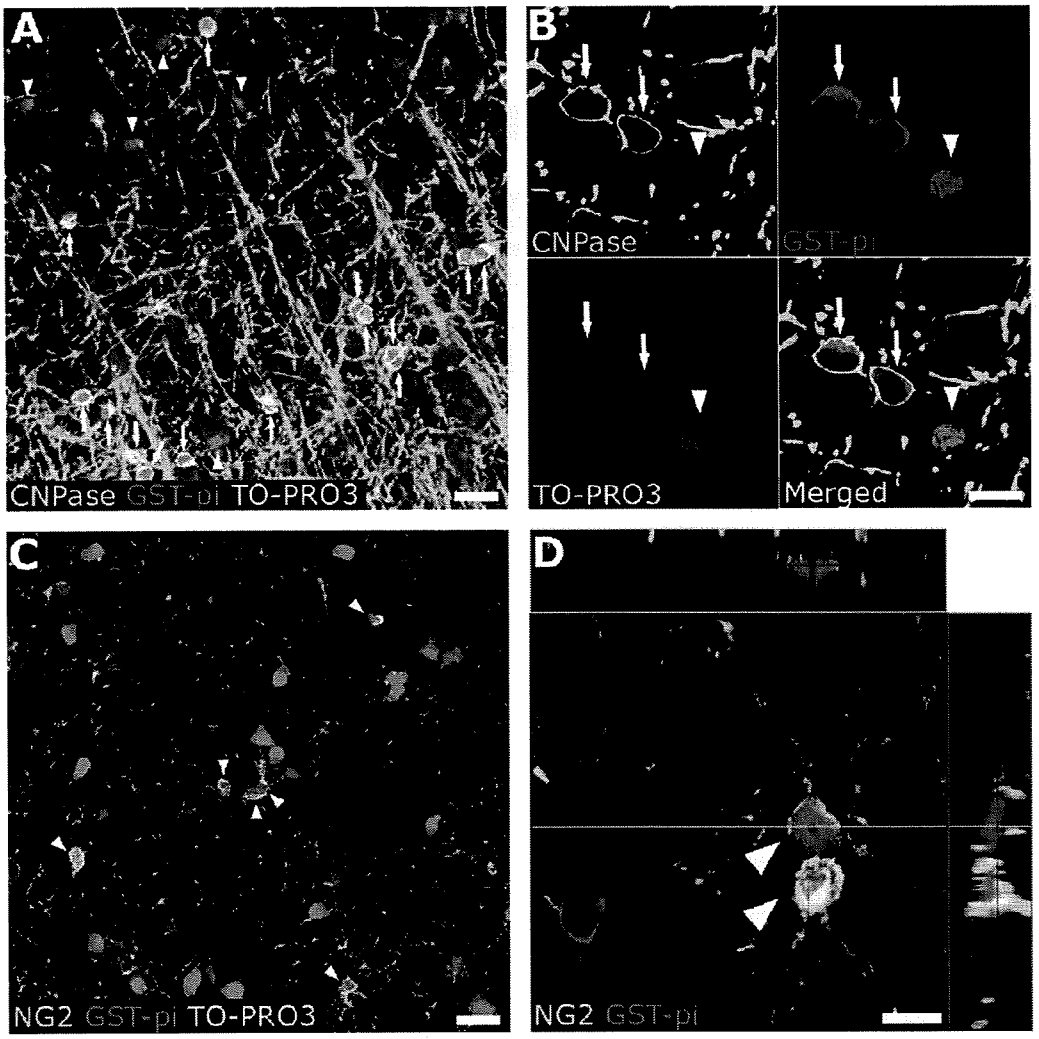

Fig. 2. Phenotypes of GST-pi (+) cells in cerebral cortex. (A, B) Triple staining for CNPase (green), GST-pi (red), and TO-PRO3 (blue) on images of single z-slices. Arrows, GST-pi (+)/CNPase (+) cells; arrowheads, GST-pi (+)/CNPase (−) cells. (C) Triple staining for NG2 (green), GST-pi (red), and TO-PRO3 (blue) on an image of a single z-slice. Arrowheads, GST-pi (+)/NG2 (+) cells. (D) A single-z-slice image with ortho-image of confocal z-stacks (upper and right panels in D) of cells double-labeled (arrowheads) with NG2 (green) and GST-pi (red). Scale bars=20 μm (A, C); 10 μm (B, D).

tified as CNPase (Watanabe et al., 2006). These findings suggested that approximately 70% of GST-pi (+) cells are CNPase (+) and RIP (+) mature oligodendrocytes, while the remaining (approximate 30%) of GST-pi (+) cells were not mature oligodendrocytes. Analysis of the intracellular distribution of GST-pi showed that all GST-pi (+)/CNPase (+) cells and GST-pi (+)/RIP (+) cells were GST-pi (+) C-type cells (arrows in Fig. 2B), whereas a majority of GST-pi (+)/CNPase (−) cells were GST-pi (+) N-type cells (arrowhead in Fig. 2B).

In the adult brain, oligodendrocyte progenitor cells (OPCs) have been identified as NG2-containing cells (Nishiyama et al., 1997; Dawson et al., 2000, 2003). We therefore next performed immunofluorescence staining for GST-pi and NG2 to examine the phenotype of residual GST-pi (+) cells containing neither CNPase nor RIP. In the cortex, all NG2 (+) cells observed in this study were GST-pi (+) cells, and 28.5±2.3% (mean±S.D., n=1384 cells from three animals) of GST-pi (+) cells were immunoreactive for NG2 (arrowheads in Fig. 2C); in addition, 77.2±3.1% (mean±S.D., n=498 cells from three animals) of GST-pi (+)/NG2 (+) cells were GST-pi (+) N-type cells (arrowheads in Fig. 2D) and 22.8±3.1% of them were GST-pi (+) C-type cells. Almost all (99.2±0.1%) GST-pi (+) N-type cells (n=376 cells from three animals) were immunopositive for NG2, while some NG2 (+)/GST-pi (+) N-type cells exhibited very weak immunoreactivity for GST-pi. No clear differences in morphology including cell size and number of processes were noted between NG2 (+)/GST-pi (+) N-type cells and NG2 (+)/GST-pi (+) C-type cells. It was confirmed that neither immunoreactivity for CNPase nor that for RIP was observed in NG2 (+) cells, as previously reported (Keirstead et al., 1998; Dawson et al., 2003).

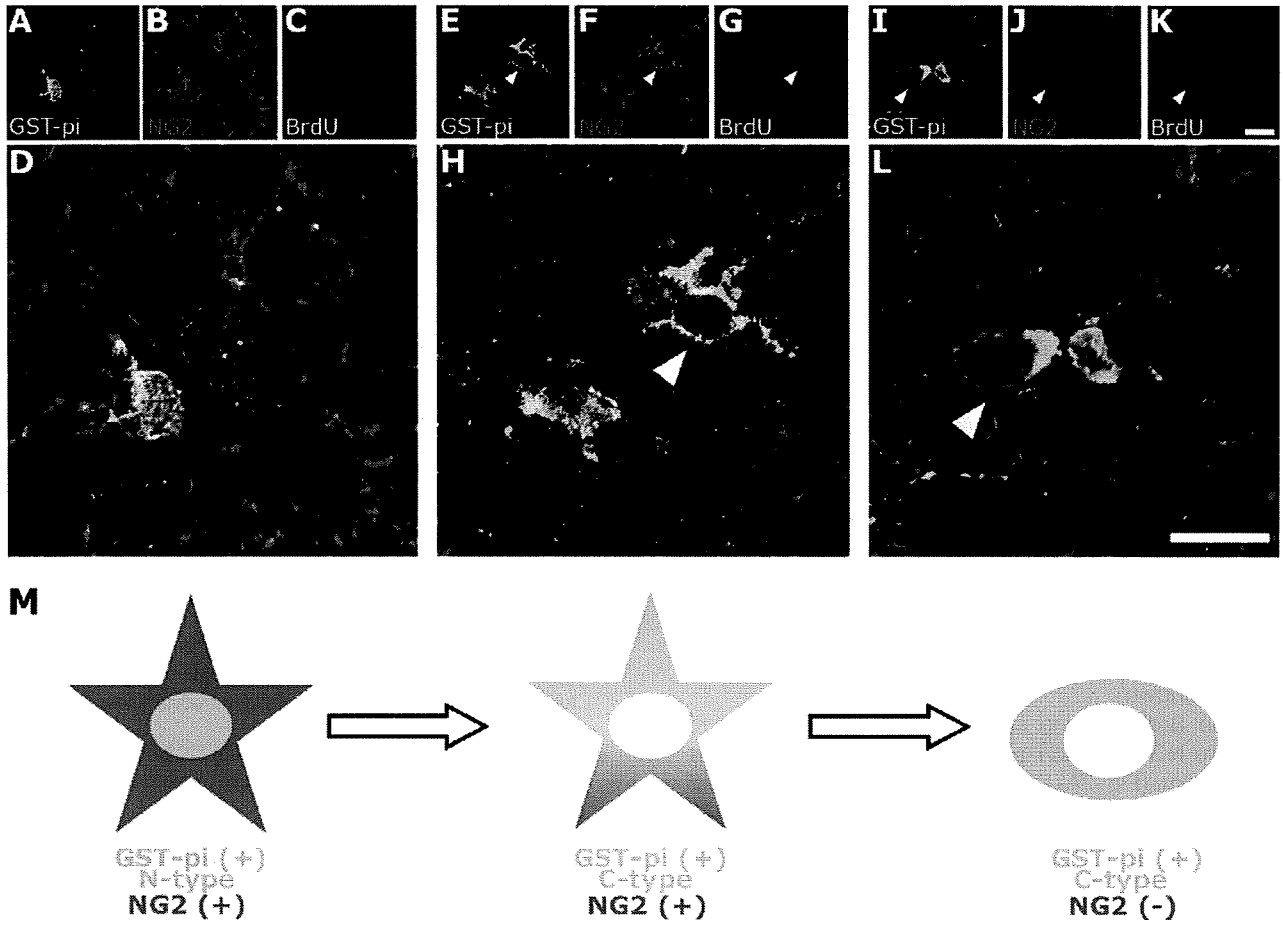

Fig. 3. GST-pi (+) N-type oligodendrocyte progenitor cells differentiated into GST-pi (+) C-type mature oligodendrocytes. (A–L) Triple immunolabeling for GST-pi (green), NG2 (red), and BrdU (blue) on images of single z-slices. An NG2 (+)/GST-pi (+) N-type BrdU-labeled cell at 2 h after BrdU injection (A–D). An NG2 (+)/GST-pi (+) N-type BrdU-labeled cell and an NG2 (+)/GST-pi (+) C-type BrdU-labeled cell (arrowheads) at 14 days after BrdU injection (E–H). An NG2 (−)/GST-pi (+) C-type BrdU-labeled cell at 28 days after BrdU injection (arrowheads in I–L). A–C, E–G, and I–K are at the same magnification; scale bar=10 μm (K). D, H, and L are at the same magnification; scale bar=10 μm (L). (M) Schema of pattern of immunoreactivity for GST-pi (green) and NG2 (red) in oligodendrocyte-lineage cells.

Nuclear GST-pi (+) (N-type) cells differentiate into cytoplasmic GST-pi (+) (C-type) cells

To determine whether GST-pi (+) N-type cells differentiate into GST-pi (+) C-type mature oligodendrocytes *in vivo*, triple-immunofluorescence staining for BrdU, GST-pi, and NG2 was performed using brain sections taken at several time points after a single injection of BrdU. At 2 h after BrdU injection, 94.7±1.9% of BrdU-labeled cells (mean± S.D., n=134 cells from three animals) were NG2 (+)/GST-pi (+) N-type cells (Fig. 3A–D). Almost all Ki67 (+) cells corresponded to GST-pi (+) N-type cells (data not shown), indicating that these cells were the major proliferating cell population. At 14 days after injection, in addition to BrdU-labeled NG2 (+)/GST-pi (+) N-type cells (82.8±4.6%; mean±S.D., n=201 cells from three animals), 9.7±2.0% (mean±S.D., n=201 cells from three animals) of BrdU-labeled cells were NG2 (+)/GST-pi (+) C-type cells (arrowheads in Fig. 3E–H). However, no NG2 (−)/GST-pi (+) C-type cells were observed among BrdU-labeled cells. These findings suggest that NG2 (+)/GST-pi (+) C-type cells are at the intermediate-differentiation stage in the oligodendrocyte lineage. At 28 days after injection, in addition to BrdU-labeled NG2 (+)/GST-pi (+) N-type cells (74.0±4.0% of BrdU-labeled cells, mean± S.D., n=227 cells from three animals) and NG2 (+)/GST-pi (+) C-type cells (11.8±2.0% of BrdU-labeled cells, mean±S.D., n=227 cells from three animals), 8.5±1.2% of BrdU-labeled cells (mean±S.D., n=227 cells from three animals) were NG2 (−)/GST-pi (+) C-type cells (arrowheads in Fig. 3I–L). These findings indicate that GST-pi (+) N-type cells differentiate into GST-pi (+) C-type mature oligodendrocytes.

DISCUSSION

It has been reported that GST-pi is expressed in carbonic anhydrase- or CNPase-positive mature oligodendrocytes in the adult rodent brain (Cammer et al., 1989; Tansey and Cammer, 1991). In the present study, we found, in the cerebral cortex of adult rats, that approximately 70% of GST-pi (+) cells were CNPase (+) and RIP (+) mature oligodendrocytes (Fig. 2A and B), while the residual 30% were NG2 (+) oligodendrocyte progenitor cells expressing

neither CNPase nor RIP (Fig. 2B and C). Although GST-pi has been used as a specific marker protein for identification of mature oligodendrocytes (Tanaka et al., 2003; Mason et al., 2004; Gotts and Chesselet, 2005), our findings indicate that careful usage of it will be necessary.

We demonstrated that two types of GST-pi (+) cells exhibited different subcellular localizations of GST-pi protein; mature oligodendrocytes contained GST-pi protein mainly in the cytoplasm (C-type cells) (Fig. 1B and arrow in Fig. 1C), while progenitor cells contained the protein mainly within the nucleus (N-type cells) (arrowhead in Fig. 1C). Even on confocal laser microscopy, it was occasionally difficult to distinguish between the N-type and C-type cells at low magnification (Fig. 2C). However, use of the profile mode of confocal laser microscope software at high magnification permitted identification of such cell types on the basis of fluorescence intensity.

These findings were demonstrated by immunohistochemical studies with both monoclonal and polyclonal anti-GST-pi antibodies. Furthermore, we confirmed by Western blotting that both monoclonal and polyclonal antibodies recognized only 23 kDa GST-pi protein in tissue homogenates from cerebral cortex of adult rats (data not shown).

In the BrdU-labeling study, almost all BrdU-labeled cells were NG2 (+)/GST-pi (+) N-type cells at 2 h after BrdU injection (Fig. 3A–D). At 14 days after injection, NG2 (+)/GST-pi (+) C-type cells began to appear besides NG2 (+)/GST-pi (+) N-type cells among BrdU-labeled cells (arrowheads in Fig. 3E–H). At 28 days after injection, NG2 (−)/GST-pi (+) C-type cells could be observed among BrdU-labeled cells in addition to the types of cells observed at 14 days (arrowheads in Fig. 3I–L). These findings indicate that NG2 (+)/GST-pi (+) N-type cells differentiate into mature oligodendrocytes, which are NG2 (−)/GST-pi (+) C-type cells, through intermediate cells, which are NG2 (+)/GST-pi (+) C-type cells (Fig. 3M).

Nuclear expression of GST-pi has been reported in human uterine cancer (Shiratori et al., 1987), human metastatic neuroblastoma (Hall et al., 1994), and human glioma (Ali-Osman et al., 1997). These observations suggest that nuclear GST-pi expression is related to cellular proliferation. Indeed, in the present study, only NG2 (+)/GST-pi (+) N-type cells exhibited proliferative activity among GST-pi (+) cells in adult cortex (Fig. 3A–D). Although the functional significance of translocation of GST-pi protein from the cell nucleus to the cytoplasm remains to be determined, observation of the intracellular location of these markers permits identification of the stages of differentiation of oligodendrocyte lineage cells.

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REFERENCES

Abramovitz M, Homma H, Ishigaki S, Tansey F, Cammer W, Listowsky I (1988) Characterization and localization of glutathione-S-transferases in rat brain and binding of hormones, neurotransmitters, and drugs. J Neurochem 50:50–57.

Abramovitz M, Listowsky I (1987) Selective expression of a unique glutathione S-transferase Yb3 gene in rat brain. J Biol Chem 262:7770–7773.

Ali-Osman F, Brunner JM, Kutluk TM, Hess K (1997) Prognostic significance of glutathione S-transferase pi expression and subcellular localization in human gliomas. Clin Cancer Res 3:2253–2261.

Board PG, Baker RT, Chelvanayagam G, Jermiin LS (1997) Zeta, a novel class of glutathione transferases in a range of species from plants to humans. Biochem J 328(Pt 3):929–935.

Board PG, Coggan M, Chelvanayagam G, Easteal S, Jermiin LS, Schulte GK, Danley DE, Hoth LR, Griffor MC, Kamath AV, Rosner MH, Chrunyk BA, Perregaux DE, Gabel CA, Geoghegan KF, Pandit J (2000) Identification, characterization, and crystal structure of the omega class glutathione transferases. J Biol Chem 275: 24798–24806.

Cammer W, Tansey F, Abramovitz M, Ishigaki S, Listowsky I (1989) Differential localization of glutathione-S-transferase Yp and Yb subunits in oligodendrocytes and astrocytes of rat brain. J Neurochem 52:876–883.

Cammer W, Zhang H (1992) Localization of Pi class glutathione-S-transferase in the forebrains of neonatal and young rats: evidence for separation of astrocytic and oligodendrocytic lineages. J Comp Neurol 321:40–45.

Dawson MR, Levine JM, Reynolds R (2000) NG2-expressing cells in the central nervous system: are they oligodendroglial progenitors? J Neurosci Res 61:471–479.

Dawson MR, Polito A, Levine JM, Reynolds R (2003) NG2-expressing glial progenitor cells: an abundant and widespread population of cycling cells in the adult rat CNS. Mol Cell Neurosci 24:476–488.

Gotts JE, Chesselet MF (2005) Migration and fate of newly born cells after focal cortical ischemia in adult rats. J Neurosci Res 80:160–171.

Hall AG, McGuckin AG, Pearson AD, Cattan AR, Malcolm AJ, Reid MM (1994) Glutathione S-transferase in bone marrow metastases of disseminated neuroblastoma. J Clin Pathol 47:468–469.

Johnson JA, El Barbary A, Kornguth SE, Brugge JF, Siegel FL (1993) Glutathione S-transferase isoenzymes in rat brain neurons and glia. J Neurosci 13:2013–2023.

Kataoka Y, Tamura Y, Takamori Y, Cui Y, Yamada H (2006) Perineuronal germinal cells in the rat cerebral cortex. Med Mol Morphol 39:28–32.

Keirstead HS, Levine JM, Blakemore WF (1998) Response of the oligodendrocyte progenitor cell population (defined by NG2 labelling) to demyelination of the adult spinal cord. Glia 22:161–170.

Li NQ, Reddanna P, Thyagaraju K, Reddy CC, Tu CP (1986) Expression of glutathione S-transferases in rat brains. J Biol Chem 261:7596–7599.

Mannervik B, Alin P, Guthenberg C, Jensson H, Tahir MK, Warholm M, Jornvall H (1985) Identification of three classes of cytosolic glutathione transferase common to several mammalian species: correlation between structural data and enzymatic properties. Proc Natl Acad Sci U S A 82:7202–7206.

Mason JL, Toews A, Hostettler JD, Morell P, Suzuki K, Goldman JE, Matsushima GK (2004) Oligodendrocytes and progenitors become progressively depleted within chronically demyelinated lesions. Am J Pathol 164:1673–1682.

Meyer DJ, Coles B, Pemble SE, Gilmore KS, Fraser GM, Ketterer B (1991) Theta, a new class of glutathione transferases purified from rat and man. Biochem J 274(Pt 2):409–414.

Meyer DJ, Thomas M (1995) Characterization of rat spleen prostaglandin H D-isomerase as a sigma-class GSH transferase. Biochem J 311(Pt 3):739–742.

Nishiyama A, Yu M, Drazba JA, Tuohy VK (1997) Normal and reactive NG2+ glial cells are distinct from resting and activated microglia. J Neurosci Res 48:299–312.

Paxinos G, Watson C (1998) The rat brain in stereotaxic coordinates, 4th ed. San Diego: Academic Press.

Pemble SE, Wardle AF, Taylor JB (1996) Glutathione S-transferase class kappa: characterization by the cloning of rat mitochondrial GST and identification of a human homologue. Biochem J 319(Pt 3):749–754.

Shiratori Y, Soma Y, Maruyama H, Sato S, Takano A, Sato K (1987) Immunohistochemical detection of the placental form of glutathione S-transferase in dysplastic and neoplastic human uterine cervix lesions. Cancer Res 47:6806–6809.

Takamori Y, Tamura Y, Kataoka Y, Cui Y, Seo S, Kanazawa T, Kurokawa K, Yamada H (2007) Differential expression of nuclear lamin, the major component of nuclear lamina, during neurogenesis in two germinal regions of adult rat brain. Eur J Neurosci 25:1653–1662.

Tamura Y, Kataoka Y, Cui Y, Yamada H (2004) Cellular proliferation in the cerebral cortex following neural excitation in rats. Neurosci Res 50:129–133.

Tanaka K, Nogawa S, Suzuki S, Dembo T, Kosakai A (2003) Upregulation of oligodendrocyte progenitor cells associated with restoration of mature oligodendrocytes and myelination in peri-infarct area in the rat brain. Brain Res 989:172–179.

Tansey FA, Cammer W (1991) A pi form of glutathione-S-transferase is a myelin- and oligodendrocyte-associated enzyme in mouse brain. J Neurochem 57:95–102.

Terrier P, Townsend AJ, Coindre JM, Triche TJ, Cowan KH (1990) An immunohistochemical study of pi class glutathione S-transferase expression in normal human tissue. Am J Pathol 137: 845–853.

Theodore C, Singh SV, Hong TD, Awasthi YC (1985) Glutathione S-transferases of human brain. Evidence for two immunologically distinct types of 26500-Mr subunits. Biochem J 225:375–382.

Watanabe M, Sakurai Y, Ichinose T, Aikawa Y, Kotani M, Itoh K (2006) Monoclonal antibody Rip specifically recognizes 2′,3′-cyclic nucleotide 3′-phosphodiesterase in oligodendrocytes. J Neurosci Res 84:525–533.

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