Developmental Shift in Bidirectional Functions of Taurine-Sensitive Chloride Channels during Cortical Circuit Formation in Postnatal Mouse Brain

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Received 29 September 2003; accepted 3 November 2003

ABSTRACT: **Taurine (2-aminoethanesulfonic acid) is the most abundant free amino acid in the developing mammalian cerebral cortex, however, few studies have reported its neurobiological functions during development. In this study, by means of whole-cell patch-clamp recordings, we examined the effects of taurine on chloride channel receptors in neocortical neurons from early to late postnatal stages, which cover a critical period in cortical circuit formation. We show here that taurine activates chloride channels in cortical neurons throughout the postnatal stages examined (from postnatal day 2 to day 36). The physiological effects of taurine changed from excitatory to inhibitory due to variations in the intracellular Cl^- concentration during development. An antagonist blocking analysis also demonstrated a developmental shift in the receptor target of taurine, from glycine receptors to $GABA_A$ receptors. Taken together, these results may reflect genetically programmed, bidirectional functions of taurine. At the early developmental stage, taurine acting on glycine receptors would serve to promote cortical circuit formation. As cortical circuit has to be regulated in the later stages, taurine would serve as a safeguard against hyperexcitable circuit.** © 2004 Wiley Periodicals, Inc. J Neurobiol 60: 166–175, 2004

Keywords: **circuit formation; depolarization; gramicidin perforated-patch recording; neuro-protective function; whole-cell patch recording**

INTRODUCTION

Taurine (2-aminoethanesulfonic acid) is one of the most abundant free amino acids in the developing nervous system (Huxtable, 1989). In mammals, sufficient taurine for embryonic development is received from the mother via the placenta, while neonates obtain taurine from mothers' milk (Sturman et al., 1977; Sturman, 1981; Chesney, 1985). Taurine is structurally similar to the inhibitory transmitter gamma-aminobutyric acid (GABA) and glycine. It has been shown that taurine interacts both GABA- (Malminen and Kontro, 1986, 1987) and glycine-receptors (Kontro and Oja, 1987a, b), and induces chloride-currents in neurons (Linne et al., 1996; Ye et al., 1997; Flint et al., 1998; Puopolo et al., 1998; del Olmo et al., 2000; Mori et al., 2002).

At the developing neocortex of the early developmental stage, nonsynaptically released taurine activates glycine-receptors (Flint et al., 1998). In a previous study, we have demonstrated that the taurine-dependent activation of glycine-receptors contributes to Ca^{2+} influx via synaptic activation of NMDARs (N-methyl-D-aspartate receptors) (Miyakawa et al., 2002). Crair and Malenka (1995) elegantly demonstrated that synaptic activation of NMDARs is criti-

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Contract grant sponsor: Grant-in Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

Contract grant sponsor: The Food Science Institute Foundation (T.H.).

Published online 16 April 2004 in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/neu.20003

cally important for the developmental plasticity of cortical circuitry. Although, in a mature cortical neuron, the voltage-dependent block of NMDARs was released by the activation of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropimate (AMPA)-type glutamate-receptors, in an immature neuron of the developing neocortex, the expression levels of AMPA receptors would not be enough to activate NMDARs (Liao et al., 1995; Isaac et al., 1997). Ben-Ari et al. first demonstrated that the depolarizing action of chloride channels compensates for the lack of AMPA receptors in an immature neuron of developing hippocampus (Ben-Ari et al., 1989, 1997). At the developing neocortex, the neurochemical machinery for the activation of NMDAR still remained unclear. The major aim of this study is to ascertain our hypothesis that taurine, which is present at extremely high levels in the developing neocortex, activates chloride receptors then depolarizes the responding immature neuron.

In the present study, the properties of taurine-sensitive receptor channels in neocortical neurons from early to late postnatal stages have been examined by means of whole-cell patch-clamp recordings. To evaluate whether the opening of taurine-sensitive receptor channels would lead to depolarization or hyperpolarization of the responding neuron, gramicidin-perforated patch-clamp recordings (Owens et al., 1996) were also carried out. The physiological effects of taurine changed from excitatory to inhibitory due to variations in the intracellular Cl^- concentration during development. From these observations, we have discussed the developmental shift in the receptor activation of taurine-sensitive chloride channels in neurons of the postnatal cerebral cortex.

METHODS

Animals

Pregnant or postnatal Institute of Cancer Research (ICR) mice were purchased from Sankyo Laboratory Service (Tokyo, Japan). The day of birth was defined as postnatal day 0 (P0). All experiments were carried out in accordance with the guidelines for Animal Experiments of the Graduate School of Frontier Sciences, The University of Tokyo.

Preparation of Acute Living Slices

ICR mice were deeply anaesthetized by hypothermia (early developmental stage (P2–P5) or ethyl ether (in excess of P6), respectively, prior to decapitation. Brains were then quickly removed and placed in ice-cold low-Ca^{2+} artificial cerebrospinal fluid (ACSF) (composition in mM: 124 NaCl, 26 $NaHCO_3$, 2.5 KCl, 10 glucose, 4.5 $MgCl_2$, 0.1 $CaCl_2$) pregassed with 95% O_2 and 5% CO_2 (pH 7.4). For preparation of brain slices, brains were glued to the cutting stage of a vibrating slice cutter (DTK-1000; Dosaka, Kyoto, Japan), rostral side up, and sliced into coronal sections (400 μm) that included the somatosensory cortex. The slices were incubated at 37°C for 30 min (recovery period) in oxygenated standard ACSF (composition in mM: 124 NaCl, 26 $NaHCO_3$, 2.5 KCl, 10 glucose, 1 $MgCl_2$, 2 $CaCl_2$), then maintained at room temperature during subsequent experiments.

Patch-Clamp Recording

Slices were fixed by nylon thread to a U-shaped platinum frame and then positioned in a recording chamber (volume approximately 0.2 ml, RC-26GLP; Warner Instruments, Hamden, CT) and submerged in and superfused with oxygenated standard ACSF at a flow rate of 1–2 ml/min as described before (Fukuda et al., 2003). The slices were observed with the aid of a microscope (BX-50WI; Olympus, Tokyo, Japan), with images recorded from the light port using a chilled charge-coupled device (CCD) video camera (C5985; Hamamatsu Photonics, Hamamatsu, Japan). Patch pipettes with resistances of 5–8 MΩ were made from fire-polished borosilicate capillary glass of 1.2 mm outer diameter (1B120F-2; World Precision Instruments, Sarasota, FL), using a programmable vertical puller (P-87; Sutter Instruments, Novato, CA). The pipette solution for whole-cell patch-clamp recordings contained (mM): 140 CsCl, 1 $CaCl_2$, 2 MgATP, 0.3 NaGTP, 10 EGTA, 10 HEPES (adjusted to pH 7.2 with CsOH).

The pipette solution for gramicidin perforated patch recording contained (in mM): 130 KCl, 5 NaCl, 0.4 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 1.1 EGTA, and was adjusted to pH 7.2 with KOH. Gramicidin (Sigma, St. Louis, MO) was first dissolved in dimethylsulfoxide (DMSO) (Sigma) to prepare a stock solution of 2 g/L and then diluted in the pipette solution to a final concentration of 5 mg/L. Fresh gramicidin-containing solutions were prepared every 2 h. After establishment of the cell-attached configuration (1–10 GΩ seal resistance), the whole-cell mode was established with negative pressure and negative current pulses using a CEZ-2400 amplifier (Nihon-Kohden, Tokyo, Japan). Perforated patch recordings were obtained using identical methods, except that mechanical rupture of the membrane was omitted. The progress of perforation was evaluated by monitoring the decrease in the membrane resistance (Owens et al., 1996).

Recordings were started after the membrane resistance had stabilized; usually 10–15 min after the high-resistance seal had been established. During the recordings, the membrane holding potential was set to −60~−70 mV. Data were recorded at room temperature with a CEZ-2400 amplifier. The membrane current was sampled on-line at 4 kHz (PowerLab; AD Instruments, Grand Junction, CO) after filtering at 2 kHz. Signals were recorded with Chart 4.0 software (AD Instruments) and stored on the hard disk of a

personal computer for later off-line analysis with Igor Pro 4.01 software (WaveMatrics, Lake Oswego, OR).

Drug Application

In some experiments, agonists were applied to one particular neuron using a "Y-tube" system (Greenfield and Macdonald, 1996; also kindly instructed by Dr. Nabekura, Kyushu University). In all other cases, agonists were pressure-applied locally using a PicoPump (PV820; World Precision Instruments) via a glass pipette positioned about 50 μm from the soma of the recorded cell. Antagonists were bath-applied in all experiments.

Patch-Clamp Data Analyses

Dose-Response Curves. Dose-response relationships for taurine were fitted to the Hill equation as follows:

$$I = I_{max}/1 + (EC_{50}/[\text{taurine}]^n)$$

where I is the peak current measured for a given taurine concentration, I_{max} the current at the maximal taurine concentration, EC_{50} the concentration of taurine required for a half-maximal response, and n the Hill coefficient (slope factor). Analysis of dose-response relationships was carried out using Origin 7.0 software (Demo) (OriginLab, Northampton, MA).

Calculation of E_{Cl} and the Intracellular Cl^- Concentration ($[Cl^-]_i$). To calculate the equivalent potential of ion A, the Nernst equation is employed:

$$E_A = \frac{RT}{FZ} \ln \frac{[A]_o}{[A]_i}$$

where R is the thermodynamic gas constant (8.31 J/mol/K), T the absolute temperature, F the Faraday Constant (96,500 C/mol), Z the valence of the ion (in the case of Cl^-, -1), and $[A]_o$ and $[A]_i$ the outside and inside concentration of ion A, respectively. In this study, I–V data were fitted by a line, with the resulting Y-intercept defined as E_{Cl}. $[Cl^-]_i$ was calculated by substituting the value of E_{Cl}, as well as 300 K for T, -1 for Z, and 133.5 mM for $[Cl^-]_o$ (ACSF composition) to the Nernst equation.

Immunocytochemistry

Mouse brains at P5 and P33 were fixed by transcardial perfusion with phosphate-buffered saline (PBS; in mM: 137 NaCl, 8.1 Na_2PO_4, 2.68 KCl, 1.47 KH_2PO_4), followed by 4% paraformaldehyde in PBS. In each case, the brain was removed and post-fixed with 4% paraformaldehyde at 4°C overnight, followed by treatment in 30% sucrose in Tris-buffered saline (TBS; in mM: 137 NaCl, 2.68 KCl, 25 Tris) overnight. Post-fixed brains were embedded in Optimal Cutting Temperature (O.C.T.) compound (Sakura, Tokyo, Japan) and frozen at −80°C (Okada et al., 2003). Frozen blocks were sliced into 40 μm-thick sections using a cryostat (MICRON, Walldorf, Germany), and then preserved in cryoprotectant solution (30% ethylene glycol and 30% glycerol in 0.05 M phosphate buffer) at −20°C. Thereafter, sections were first placed in TBS for 10 min to wash out the cryoprotectant and then blocked with blocking buffer [5% normal goat serum (NGS) in 0.3% Triton-TBS] for 30–60 min at room temperature. Free-floating sections were incubated for 3 days at 4°C with monoclonal anti-glycine receptor (mouse IgG; 1:100 dilution; Alexis, Lausen, Switzerland) and anti-sodium, potassium, chloride cotranporter 1 (NKCC1) (Plotkin et al., 1997) (rabbit IgG; 1:100 dilution; Chemicon, Temecula, CA) in modified blocking solution (5% NGS in 0.1% Triton-TBS). After three washings in TBS (10, 15, and 15 min), sections were incubated with fluorochromo-conjugated secondary antibodies, Alexa 488 (anti-rabbit IgG; 1:1000 dilution, Molecular Probes, Eugene, OR) and RRX (anti-mouse IgG; 1:200 dilution; Jackson Immunoresearch, West Grove, PA), dissolved in the modified blocking solution at room temperature for 2 h, and then washed in TBS. The stained sections were aligned on glass slides and incubated for 10 min in 4′,6-diamidino-2-phenylindole (DAPI) (1:1000 dilution; Sigma) dissolved in 0.1% Triton-TBS, and then sealed by Immu-mount (Shandon, Pittsburgh, PA) with 2% 1,4-diazabicyclo[2.2.2]octane (DABCO) (Sigma). Stained sections were analyzed with a confocal microscope (TCS SP2; Leica, Mannheim, Germany) (Koketsu et al. 2003).

RESULTS

Response of Cortical Neurons to Taurine during the Postnatal Stages

Taurine-induced currents in supragranular layer neurons (layers II–IV) were measured by means of whole-cell patch-clamp recordings. Figure 1(A) shows that current activation in a P6 neuron occurred when taurine was employed at a concentration in excess of 0.3 mM. In general, while no evidence of desensitization was observed in P2–7 neurons for taurine concentrations up to 1 mM, slight desensitization was observed at concentrations above 1 mM. Currents were clearly desensitized at taurine concentrations in excess of 3 mM. Average dose-response values for five P2–7 neurons were fitted by a Hill equation, for which the calculated half-maximal concentration (EC_{50}) and Hill coefficient were 7.7 mM and 0.94, respectively [Fig. 1(B)]. Recorded neurons in the supragranular layer which stained with lucifer yellow possessed apical dendrites and seemed to be typical of pyramidal neurons [Fig. 1(C)].

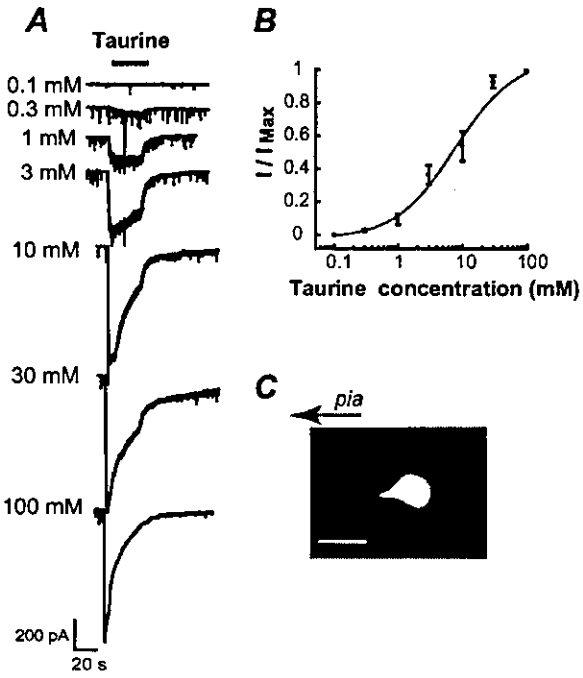

Figure 1 Responses of early developmental stage (P6) neurons to activation by taurine. (A) Currents measured in a P6 neocortical neuron in response to exposure to different concentrations of taurine. Recordings made in whole-cell patch-clamp mode. Note that at lower concentrations (~1 mM) taurine evoked electrical responses without desensitization. Comparable results were obtained from four other neurons and their recording data were utilized for subsequent analysis. (B) Dose-response curve for taurine in early developmental stage (P2–7) from the recordings of five neurons, which were obtained from five brain slices of three mice. The data were fitted by a Hill equation for which the EC_{50} and Hill coefficient were 7.7 mM and 0.94, respectively. (C) Representative morphology of a neuron typical of one from which recordings were made, visualized here by lucifer yellow staining. Scale bar: 20 μm.

Responses of cortical neurons to taurine were further analyzed for later developmental stages. As shown in Figure 2, taurine triggered currents in P27–36 neocortical neurons. Average dose-response values for four P30 neurons were fitted by a Hill equation, for which the calculated half-maximal concentration (EC_{50}) and Hill coefficient were 3.9 mM and 0.95, respectively [Fig. 2(B)]. The morphology of recorded neurons was visualized with the aid of lucifer yellow injection; an example of one of these neurons is shown in Figure 2(C) which exhibits several processes and pyramidal morphology. Cortical neurons at a more developed stage, such as in young adults, were also tested for taurine-evoked responses. As shown in Figure 3, taurine evokes currents in cortical neurons from 8-week-old mice at concentrations as low as 0.3 mM. This is the first demonstration, to our knowledge, of taurine triggering an electrical response in adult cortical neurons, and implies that taurine could act as a neuromodulator in the adult cerebral cortex.

Developmental Shift of Taurine-Sensitive Receptor Channels from GlyR to $GABA_AR$

The current response triggered by taurine application was subsequently examined in neurons from two developmental stages: the early developmental stage (P2–7) and the late developmental stage (P27–36). In order to analyze the properties of the receptor-coupled channels via which the taurine-evoked currents were conducted, pharmacological agents were subsequently used to inhibit receptors thought to be involved in the mechanism.

As mentioned in the Introduction, it has been reported that taurine acts as an agonist for glycine receptors (Flint et al., 1998; Mori et al., 2002) and $GABA_A$ receptors (Puopolo et al., 1998; del Olmo et al., 2000). On this basis, we evaluated the properties of taurine responses and the taurine-induced activation of glycine receptors and/or $GABA_A$ receptors in cortical neurons from mouse neocortex from each of the developmental stages.

To identify the receptor-coupled channels mediating these currents, specific receptor antagonists were used in whole-cell patch-clamp recordings. As shown in Figure 4, the currents evoked in a P5 neuron by taurine application (2 mM) were mostly blocked by strychnine, a selective antagonist of glycine receptors. The remaining small residual current was completely blocked by the addition of bicuculline methiodide (BMI), a selective antagonist of $GABA_A$ receptors. In control experiments, it was confirmed that the strychnine dosage used—3 μM—completely inhibited the glycine response but did not disturb the GABA response, and similarly that the BMI dose used (100 μM) completely blocked the GABA response. Although taurine activated $GABA_A$ receptors as well as glycine receptors in this P5 neuron, the response was largely mediated by glycine receptors.

In cortical neurons from the late developmental stage (P27–36), the taurine response was only partially inhibited by strychnine, with the remainder completely inhibited by the addition of bicuculline methiodide (Fig. 5). Table 1 summarizes the results obtained from neurons in the early and late developmental stages. The component that was inhibited by the application of strychnine is defined as "GlyR," and the residual component, which is completely in-

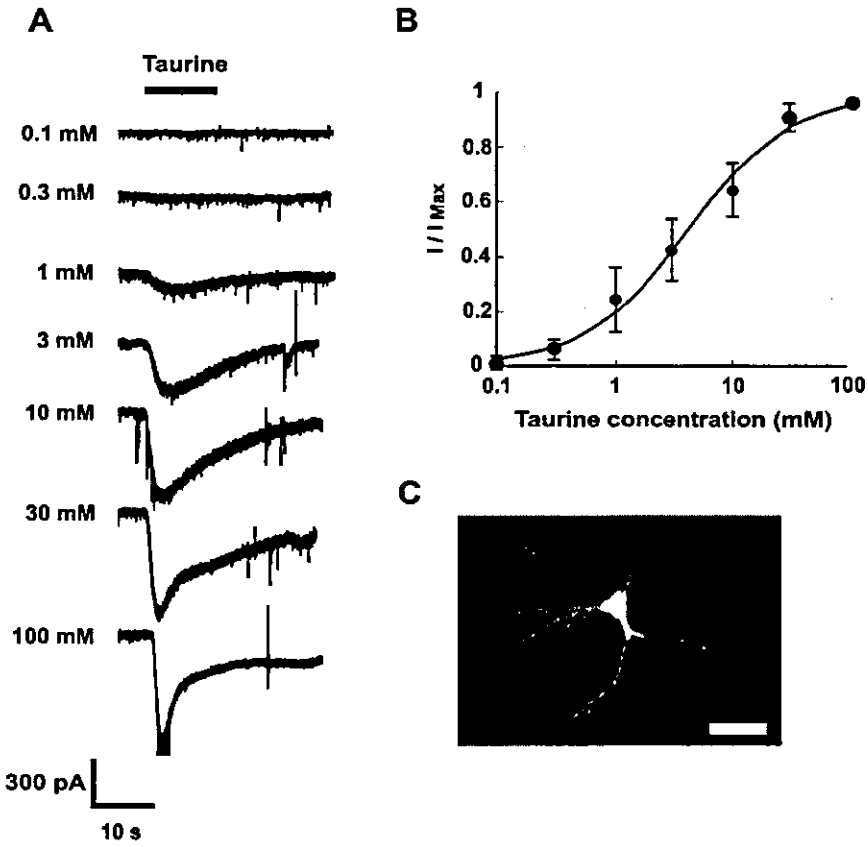

Figure 2 Responses of late developmental stage (P30) neurons to activation by taurine. (A) Currents measured in a P30 neocortical neuron in response to exposure to different concentrations of taurine. Recordings made in whole-cell patch-clamp mode. Comparable results were obtained from three other neurons and their recording data were utilized for subsequent analysis. (B) Dose-response curve for taurine in a late developmental stage (P30) from the recordings of four neurons, which were obtained from three brain slices of three mice. The data were fitted by a Hill equation for which the EC_{50} and Hill coefficient were 3.9 mM and 0.95, respectively. (C) Representative morphology of a neuron typical of one from which recordings were made, visualized here by lucifer yellow staining. Scale bar: 20 μm.

hibited by the additional application of bicuculline methiodide, is defined as "$GABA_AR$." The average taurine response evoked in the absence of any inhibitor for cells at each developmental stage was designated as the 100% value, and each component (GlyR or $GABA_AR$) then normalized to this value. The results clearly show a developmental shift in the taurine target, from the glycine receptor to the $GABA_A$ receptors.

Developmental Stages Determine the Physiological Functions of Taurine

We studied the physiological effects of taurine on the taurine-sensitive receptor channels. Given that taurine acts on Cl^- ion channels, the gramicidin perforated patch-clamp method was employed to monitor the physiological action of taurine in maintaining the intracellular Cl^- concentration. The effects of taurine application were observed both in the early and later developmental stages (Fig. 6). These observations were carried out under current-clamp mode, in which changes in membrane potential could be recorded in response to exposure of cortical neurons to taurine. For neurons in the early developmental stage (P2), taurine application induced a +15 mV depolarization, from −70 mV to −55 mV [Fig. 6(A)]. I–V curves were constructed by switching from current-clamp to voltage-clamp mode and then measuring current amplitudes in response to changes in holding potentials. The equilibrium potential for taurine for the neuron whose experimental results are shown in Figure 6(A) was thus found to be −42 mV, from which the intracellular Cl^- concentration was calculated from the

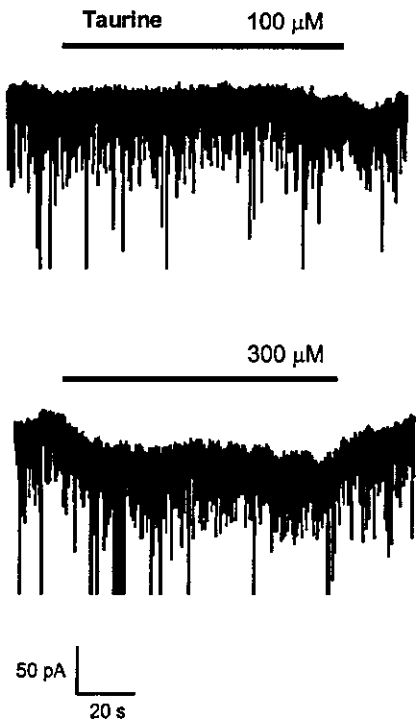

Figure 3 Electrical responses of adult neocortical neurons. Taurine evoked responses in adult neocortical neurons (8-week-old mice) when used at a concentration of 0.3 mM. Comparable results were obtained from three other adult neurons, from three brain slices of three mice.

Nernst equation to be 26.2 mM. Since taurine-evoked currents were completely inhibited by the Cl^- channel receptor antagonists strychnine and BMI (Fig. 4), the equilibrium potential for taurine (see comments above) was considered to be equal to that for Cl^-. For a neuron from the later developmental stage (P33), similar experiments were performed [Fig. 6(B)]. In this case, the membrane potential hyperpolarized by −4 mV (from −73 mV to −77 mV) in response to taurine application, which gave rise to an equilibrium potential of −86.8 mV and an intracellular Cl^- concentration of 4.62 mM.

Following on from these results, the change of intracellular Cl^- concentrations was examined for three different developmental stages (Fig. 7). Results showed a decrease of intracellular Cl^- concentration as the postnatal period progressed. We considered that the decrease in the intracellular Cl^- concentration measured in older cortical neurons could be associated with a decrease in the density of the Na^+-K-$2Cl^-$ cotransporter, NKCC1. An anti-NKCC1 antibody was thus used to enable immunohistochemical examination of the expression of this cotransporter in cortical neurons at the various developmental stages. The expression of GlyRs in developing cortical neurons was also evaluated. In neocortical neurons from animals at an early developmental stage (P5), glycine receptors were found to be densely co-expressed with NKCC1 in most cells of the neocortical layer II/III (Fig. 8). In neurons from animals at a later developmental stage (P33), cells were present in relatively sparse amounts in the cortical plate as revealed by DAPI staining, but many of the cells stained positively for the glycine

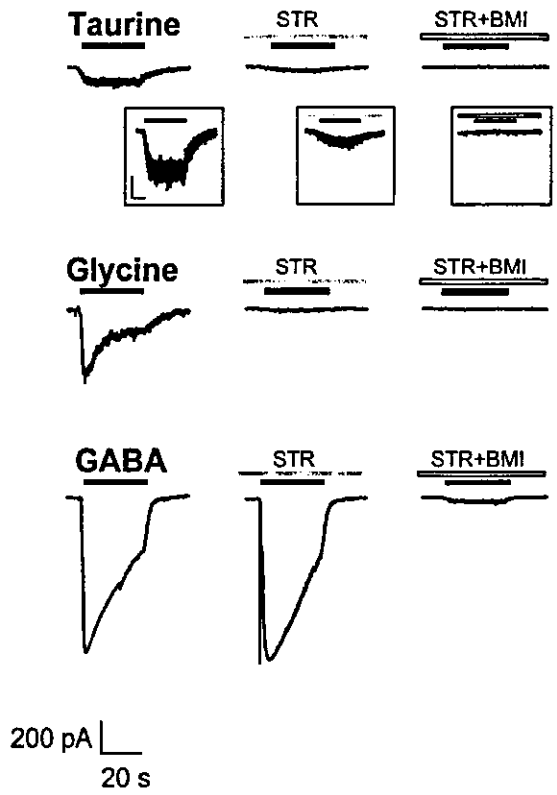

Figure 4 Effects of glycine receptor and $GABA_A$ receptor antagonists on taurine-evoked current responses in a P5 neocortical neuron. Taurine (2 mM) evokes an electrical response in a layer II/III P5 neuron. Solid bar indicates the time of taurine application. Insets show magnifications of the taurine responses. Scale bars in insets are 50 pA and 10 s. The taurine response (upper trace) was mostly inhibited by the application of strychnine (STR; 3 μM). Longer bar shows the time of inhibitor application. The residual current elicited during the taurine application was completely blocked by the addition of bicuculline methiodide (BMI; 100 μM). Middle trace shows a control experiment where the same neuron as in upper trace is activated by glycine (1 mM). This response was completely blocked by STR. The lower trace shows a control experiment, again on the same neuron, with activation by GABA (100 μM). On this occasion, the response was blocked by BMI (100 μM). These control experiments highlight the specificity of the inhibitors used in this study. Comparable results were obtained from 10 other neurons, and these recording data were utilized in Table 1.

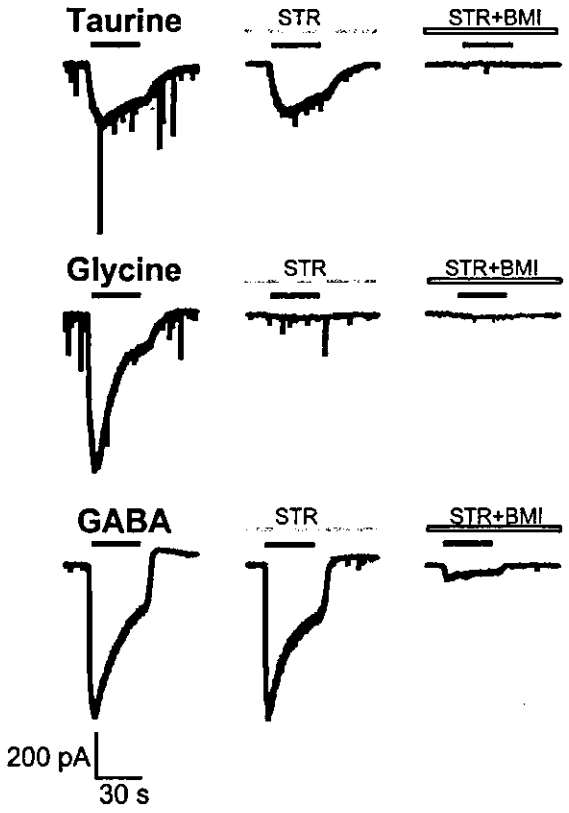

Figure 5 Effects of glycine receptor and $GABA_A$ receptor antagonists on taurine-evoked current responses in a P33 neocortical neuron. Taurine (2 mM), glycine (1 mM), and GABA (100 μM) were applied to a P33 neuron in neocortical layer II/III. Experimental conditions are the same as those described in the legend to Figure 4 . The effect of STR is less than that seen for the P5 neuron in Figure 4 . Comparable results were obtained from two other neurons, and these recording data were utilized in Table 1.

receptor (Fig. 8). NKCC1 was negatively stained in the whole slice, meaning that the co-expression of glycine receptors and NKCC1 was not observed in

Table 1 Principal Target of Taurine Shifts from Glycine Receptors to $GABA_A$ Receptors as Development Proceeds[1]

Developmental Stage	GlyR (%)	$GABA_AR$ (%)
Early stage	82.2	17.8
Late stage	27.6	72.4

[1] Early developmental stage (early stage), P2–7 (n = 11, from 11 slices of five mice); late developmental stage (late stage), P27–36 (n = 3, from three slices of two mice). The current component that was inhibited by strychnine (3 μM) was defined as the "GlyR" component of the current, with the residual current defined as the "$GABA_AR$" component. The percentages of GlyR and $GABA_AR$ are the mean values. S.E.M. of the value at each stage was 4.1% (early stage) and 4.5% (late stage).

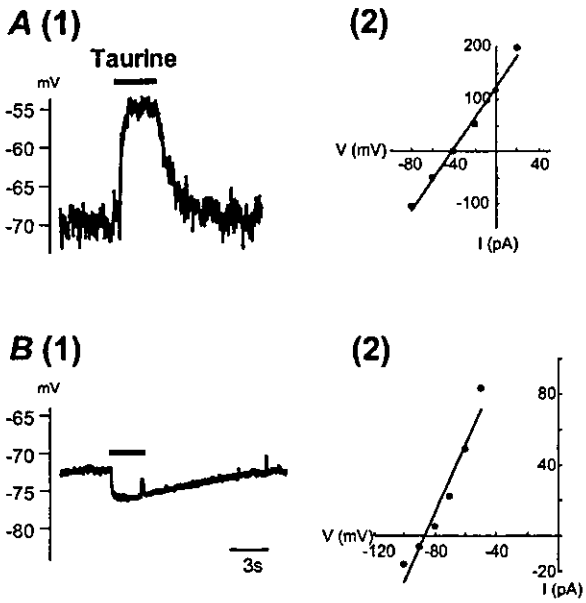

Figure 6 Two different effects of taurine as a function of developmental stage. (A) (1) Gramicidin perforated patch-clamp recording in current-clamp mode from a P2 neocortical neuron demonstrates that taurine application (10 mM) induces membrane depolarization. (2) For this neuron, E_{Cl} = −42.0 mV and $[Cl^-]_i$ = 26.2 mM as calculated by the Nernst Equation. (B) (1) Recording from a P33 neuron shows cell membrane hyperpolarization upon taurine application. (2) In this neuron, E_{Cl} = −86.8 mV and $[Cl^-]_i$ = 4.62 mM.

P33 neocortical layer II/III neurons. It can therefore be stated that the expression of NKCC1 in neurons in these experiments was limited to the early developmental stage (P2–7), which is consistent with the presence of a developmental program for regulating the intracellular Cl^- concentration in mammalian cortical neurons.

DISCUSSION

In this communication, we clearly demonstrate that taurine activates specific chloride channels in developing cortical neurons in a manner dependent on the postnatal stage of the developing brain (from P0–P36). The physiological effect of activation of the taurine-receptor channels changes from excitatory to inhibitory over the course of postnatal development due to the marked decrease in the intracellular Cl^- concentration during this time. This developmental shift occurs in parallel with the shift in the receptor-type responsible for mediating the activation by taurine, from glycine receptors to $GABA_A$ receptors. In addition, it has been re-

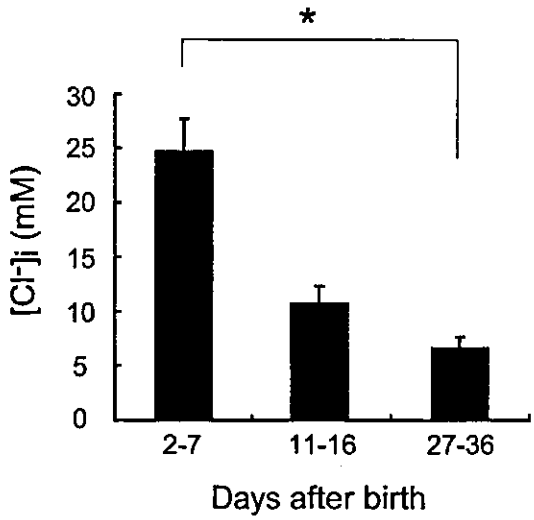

Figure 7 Decrease in intracellular Cl^- concentration during development. $[Cl^-]_i$ was estimated by means of gramicidin perforated patch-clamp recordings in developing neocortical layer II/III neurons. Numbers of trials: early stage (P2–7), $n = 35$; intermediate stage (P11–15), $n = 8$; late stage (P27–36), $n = 3$. Values are given as means ± S.E.M. $^*p < 0.05$, Student's t-test for unpaired samples.

ported that a gradual decrease in brain taurine concentration from birth is completed by the time of weaning or thereabouts in rodents and primates (Sturman, 1986). From these results, we can postulate the existence of genetically programmed, bidirectional functions of taurine in the developing cerebral cortex: at the early postnatal stage, taurine would function as a promoter of cortical circuit formation, whereas later on in postnatal development, taurine assumes the role of toning down the hyperexcitable behavior of immature cortical circuitry.

Cortical circuit formation is strictly regulated as a function of postnatal age (Feldman et al., 1999). Remarkably, thalamocortical long-term potentiation (LTP) is only observed in brain slices from P7 animals or younger (Crair and Malenka, 1995). This developmental time course is similar to the critical period for experience-dependent plasticity of thalamocortical synapses in vivo (Schlaggar et al., 1993). This critical period occurs in synchrony with the early postnatal stage (P0–P6), when the interaction between taurine and glycine receptors serves as an inducer for NMDA receptor activation by overriding the effects of voltage-dependent Mg^{2+} block (Flint et al., 1998; Miyakawa et al., 2002).

The expression of glycine receptors in cortical neurons has been reported in a series of studies. In neonates, $\alpha 2$ subunits and β subunits are expressed in cortical neurons in developing cortical plates (Malosio et al., 1991). It has been also reported that glycine receptors in neonates consist of five $\alpha 2$ subunits, expressed as a homopentamer (Langosch et al., 1988; Takahashi et al., 1992; Flint et al., 1998). In relation to later developmental stages, little information is available concerning glycine receptor expression in the neocortex. In this study, we have demonstrated for the first time, to our knowledge, the expression of glycine receptors in cortical neurons, even though at this stage taurine preferentially activates $GABA_A$ receptors rather than glycine receptors. This developmental shift in the target of taurine probably stems from a developmental shift in the subunit compositions of glycine and $GABA_A$ receptors.

We have shown, in this communication, that a taurine-induced opening of chloride channels leads to a decrease in membrane potential of neurons after the later developmental period. It has also been reported that excitotoxic amino acids such as glutamate signif-

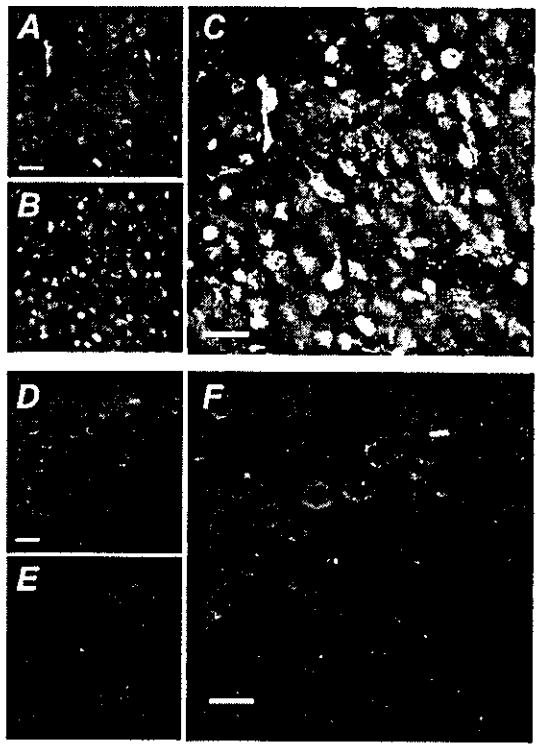

Figure 8 Expression of glycine receptors and NKCC1 in layer II/III cortical neurons of early (P5) and late (P33) developmental stages. (A–C) Images from P5 neurons. Glycine receptors (A) and Na^+-K^+-$2Cl^-$ cotransporters (NKCC1; B) were densely co-expressed in P5 neocortical neurons. (C) Merged image of A and B. (D–F) Images from P33 neurons. Glycine receptors (D) were expressed at the surface of P33 cortical neurons, but no NKCC1 expression was observed (E). (F) Merged image of A and B with DAPI staining for identification of cortical cells. Scale bars: 20 μm.

icantly stimulate the release of taurine (Saransaari and Oja, 1997; Oja and Saransaari, 2000). It is conceivable that taurine at this stage could contribute to a reduction in the membrane hyperexcitability of neuronal cortical circuitry. Indeed, GABA as the principal inhibitory neurotransmitter in the neocortex plays an important role in epilepsy (Treiman, 2001). Since the kinetics of the taurine response on $GABA_A$ receptors are quite unique when compared to the primary ligand GABA (Ye et al., 1997; del Olmo et al., 2000), taurine could therefore be able to act as a secondary ligand for $GABA_AR$ in the mammalian cerebral cortex, serving as an additional safeguard against hyperexcitability in later postnatal stages of developing cerebral cortex.

We thank Drs. Fusao Kato and Junichi Nabekura for informative discussions.

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Journal of Neuroscience Research 75:817–824 (2004)

Neural Precursor Cells Derived From Human Embryonic Brain Retain Regional Specificity

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Recent studies have revealed that neural precursor cells can be expanded not only from the subventricular zone and hippocampus but also from other regions of the human embryonic brain. To determine the regional differences of these precursor cells, we divided the brain of a 9-week-old human embryo into four parts, i.e., telencephalon, diencephalon, mesencephalon, and rhombencephalon. All cultures of the tissues yielded neurospheres, and these spheres gave rise to neurons, astrocytes, and oligodendrocytes. An analysis of clonal populations revealed that these precursor cells were multipotent, and two region-specific differences in neural precursor cells were revealed: 1) The precursor cells from the rostral part of the brain tended to proliferate faster than those from the caudal part, and 2) the precursor cells from the diencephalon and mesencephalon gave rise to more tyrosine hydoxylase (TH)-positive neurons than those from the telencephalon and rhombencephalon. When 50-day-cultured spheres were caused to differentiate, the percentage of TH-positive cells per total cell population was 1.2% for diencephalic and mesencephalic precursors, whereas it was 0.4% for telencephalic and rhombencephalic ones. Furthermore, the TH-positive cells from diencephalic and mesencephalic precursors were large, multipolar, and γ-aminobutyric acid (GABA)-negative, which suggested that these cells were midbrain dopaminergic neurons. In contrast, TH-positive cells from telencephalic and rhombencephalic precursors were small, bipolar, and GABA-positive. These results suggest that human neural precursor cells might have the potential to differentiate into a variety of cells but retain regional specificity.

Key words: dopaminergic neuron; GABA; astrocyte; differentation

Multipotent neural stem cells have been isolated from fetal and adult rodents (for review see Gage, 2000). These cells proliferate for a long period, giving rise to neurons, astrocyte, and oligodendrocytes, and, when grafted into the brain, they survive and differentiate into neurons to improve impaired function in animal models of diseases such as Parkinson's disease and ischemia. Human neural precursor cells (NPCs), namely, neural stem cells and neural progenitor cells, have also been isolated from fetal and adult brain (Svendsen et al., 1997; Vescovi et al., 1999; Carpenter et al., 1999; Ostenfeld et al., 2000; Palmer et al., 2001). These cells also give rise to neurons and glial cells in vitro and survive to differentiate into neurons in the rat brain (Fricker et al., 1999). These findings support the idea that the NPC is a good candidate for cell transplantation therapy against various neurological diseases.

In the normal rodent brain, two regions are known to be neurogenic, i.e., the subventricular zone of the lateral ventricle and the hippocampus. However, NPCs reside not only in these two areas but also in the cerebral cortex and spinal cord (for review see Gage, 2000). Therefore, for clinical application, it is important to determine whether NPCs can be expanded from all regions of the human brain. Also, the second question to be answered is whether the NPCs derived from different parts of the brain have the same characteristics.

In early human brain development, the three primary brain vesicles become subdivided into five vesicles as development continues (Langman, 1981). Forebrain (prosencephalon) is divided into the telencephalon and diencephalon. The former forms the olfactory bulbs, hippocampus, and cerebrum, the latter retina, thalamus, and hypothalamus. The midbrain (mesencephalon) continues

Contract grant sponsor: JSPS; Contract grant sponsor: MEXT; Contract grant sponsor: MHLW of Japan.

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Received 27 October 2003; Revised 1 December 2003; Accepted 2 December 2003

Published online 25 February 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.20046

to be the mesencephalon, which forms the midbrain in the adult. The hindbrain (rhombencephalon) is divided into the metencephalon and myelencephalon. The former becomes the cerebellum and pons, the latter the medulla oblongata.

Recent evidence suggests that murine and human NPCs have different potentials to proliferate and differentiate according to their region of origin (Hitoshi et al., 2002; Ostenfeld et al., 2002; Jain et al., 2003). To answer the questions posed above, we cultured dissociated cells derived from four different parts of a human embryonic brain. We present here evidence that human NPCs from different parts of the brain retain regional specificity with respect to proliferation rate and potential to give rise to tyrosine hydroxylase (TH)-positive neurons.

MATERIALS AND METHODS

Culture of Human NPCs

A human brain from a 9-week-old, therapeutically aborted embryo was obtained in accordance with the guidelines of the Committee of Medical Ethics of Kyoto University. The brain was divided into the following four pieces: telencephalon, diencephalon, mesencephalon, and rhombencephalon. Each tissue was mechanically dissociated and cultured in serum-free medium consisting of Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12; 1:1) containing N2 supplement (Gibco, Grand Island, NY), fibroblast growth factor-2 (FGF-2; 20 ng/ml), epidermal growth factor (EGF; 10 ng/ml), and leukemia inhibitory factor (LIF; 10 ng/ml). NPCs were grown as neurospheres, and the spheres were cut into small pieces with microscissors when they had become 1.0 mm in diameter.

For counting a total cell number, 1/100 volume of the spheres was picked up after being cut with microscissors and then dissociated with 0.25% trypsin to a cell suspension. The density and viability of an aliquot was checked by using trypan blue exclusion with a hemocytometer.

For differentiation, NPCs were plated on chamber slides coated with poly-L-ornithine and laminin. The medium was changed to that containing brain-derived neurotrophic factor (BDNF; 20 ng/ml) and neurotrophin-3 (NT-3; 40 ng/ml) in place of FGF-2, EGF, and LIF. Statistical analyses were conducted by ANOVA for comparison between origins of NPCs and by the Mann-Whitney U-test for the data between 50- and 150-day cultures.

Immunofluorescence Study

After 14 days in vitro, immunofluorescence staining was performed as previously described (Palmer et al., 1997). Except where indicated, all staining procedures were carried out at room temperature. Briefly, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, rinsed three times with PBS, and treated as follows. The cells were first preincubated with PBS containing 5% preimmune donkey serum and 0.3% Triton X-100 (PBST-DS) for 30 min. Then, they were incubated with primary antibodies in PBST-DS overnight at 4°C. After three washes with PBST, the cells were next incubated for 2 hr with secondary antibodies (Jackson Immunoresearch, West Grove, PA) conjugated to Texas red or cyanin-5 (diluted 1:500 in PBST-DS). The cells were then washed three times with PBST. The coverslips were transferred onto glass slides bearing 100 mM Tris, pH 8.5, containing 25% glycerol, 10% polyvinyl alcohol (Air Products), and 2.5% 1,4-diazobicyclo-[2.2.2]-octane (Sigma, St. Louis, MO). All primary antibodies were diluted to their optimum concentration (mo, mouse monoclonal; rb, rabbit polyclonal): mo anti-Map2ab, 1:250 (Sigma); mo anti-Tuj1, 1:300 (Babco); rb anti-GFAP, 1:2,000 (Chemicon, Temecula, CA); mo anti-galactocerebroside, 1:2 (H8-GalC hybridoma supernatant; a gift from O. Boegler); mo anti-O4, 1:60 (Chemicon); rb anti-TH, 1:60 (Chemicon); mo anti-GABA, 1:2,000 (Sigma). For nuclear staining, 200 ng/ml of 4′,6′-diamidino-2-phenylindole (DAPI) was added in the final wash. After immunofluorescence staining, the percentage of immunoreactive cells was evaluated by using a Fluoview FV300 laser confocal microscope (Olympus Optical Co.). For each group, the cells of 10 fields that included 100–500 cells were counted. The number of total cells was evaluated by counting DAPI-positive nucleus.

Retroviral Transduction and Cloning

The LNCE vector was constructed from LNCX (Clontech, Palo Alto, CA) by replacing the neomycin resistance gene with the hygromycin B resistance gene, and inserting the enhanced green fluorescent protein (EGFP; Clontech) coding sequence. 293T cells were transiently cotransfected with pLNCE and the packaging construct pCL (Naviaux et al., 1996) bearing amphotropic envelop by using Cellphect (Amersham Pharmacia, Picataway, NJ). After overnight incubation, the medium was replaced with DMEM/F12 supplemented with N2 supplement (Gibco), and the supernatant was collected 2 days later. The obtained virus titer was 5×10^4 cfu/ml. To generate human neural precursor populations derived from single marked cells, we treated dissociated cells with these retroviral vectors. About 5% of the cells were infected and came to be GFP-positive. Three days later, a single fluorescent cell or small spheres including only one fluorescent cell were transferred to 96-well dishes. To improve cell survival during selection with 100 μg/ml hygromycin B, we replaced half of the medium every 3 days with fresh medium supplemented with medium conditioned by human NPCs.

Southern Blot Analysis

Ten milligrams of genomic DNA were digested overnight with restriction enzymes that cut only once within the vector. The digested DNA was denatured and transferred to a nylon membrane. The filters were prehybridized overnight at 42°C in 50% deionized formamide in 6× SSC, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and salmon sperm DNA and then hybridized under the same conditions with ^{32}P-labeled EGFP cDNA. The filter was washed repeatedly at 68°C in 0.2× SSC, 0.1% SDS.

Reverse Transcription-Polymerase Chain Reaction Analysis

Total RNA from proliferating NPCs was isolated by using Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instruction. Possible contaminating genomic DNA was eliminated with DNase. SuperScript TM

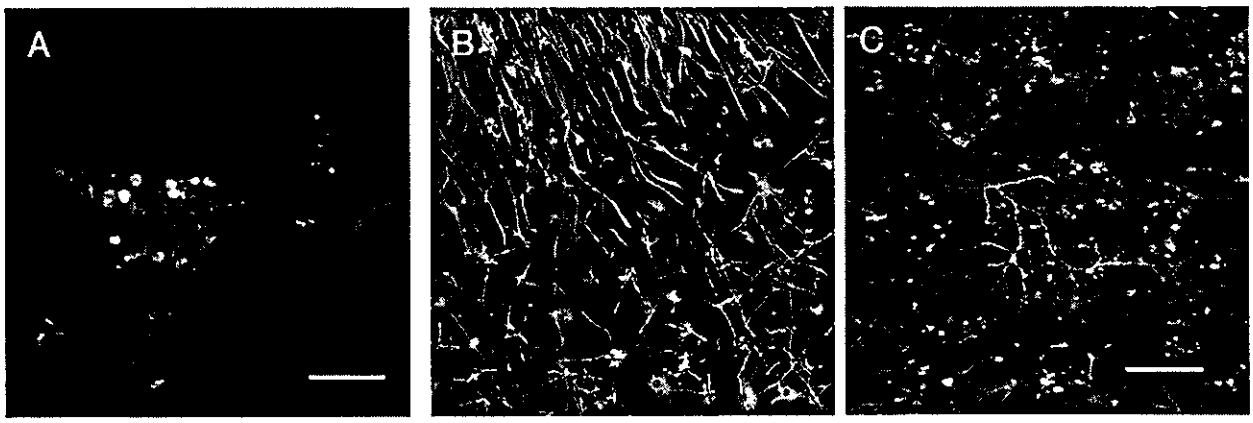

Fig. 1. Characteristics of human NPCs. As a representative population, cells derived from telencephalon are shown. The cells being cultured for 100 days as spheres were made to differentiate. **A:** Confocal micrograph of a neurosphere 24 hr after it had been plated onto a polyornithine/laminin-coated dish. Most of the cells were positive for Musashi-1 (red). A few MAP2ab-positive neurites (green) were also visible. **B,C:** Confocal micrographs of differentiated cells. Human NPCs gave rise to MAP2ab-positive neurons (green in B), GFAP-positive astrocytes (red in B,C), and a few galactocerebroside-positive oligodendrocytes (green in C). Scale bar in A = 50 μm; bar in C = 100 μm for B,C.

(Life Technologies) was used for reverse transcription with random hexamers (25 pmol) as primers in a 20-μl reaction mixture. For PCR amplification, the sets of primers shown below were used. Reactions were performed in a 25-μl reaction mixture using rTaq TM (TaKaRa), with a regimen of 20 sec at 94°C, 20 sec at 55°C, 30 sec at 72°C for 30 cycles in a thermal cycler (Perkin Elmer, Norwalk, CT). Five microliters of each reaction were analyzed by 2% (w/v) agarose gel electrophoresis. Amplified products were sequenced to rule out false positives.

Primers used for RT-PCR were as follows: Otx2, 5′-TATGCTGGCTCAACTTCCTAC-3′ and 5′-ATAATCCAAG-CAGTCAGCATT-3′; Gbx2, 5′-CTTCGCTCGTCGGGG-CTGTCC-3′ and 5′-GCCTTCACCCGTTTCCACTTG-3′; Pax6, 5′-CCAACGATAACATACCAAGCG-3′ and 5′-GG-CCTGTCTTCTCTGATTCC-3′; Emx2, 5′-CGGACAAT-GACAAGGAGACC-3′ and 5′-TGTACTGGGCGTTGTA-CTGC-3′; G3PDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′; Ptc, 5′-CAT-TGGCAGGAGGAGTTGAATTGTGG-3′ and 5′AGC-ACCTTTTGAGTGGAGTTTGGGG-3′; and Smo, 5′-GTAGTGTGGTTCGTGGTCCTC-3′ and 5′-GGT-TGGCTTGTTCTTCTGGTG-3′.

RESULTS

The intact whole brain of a 9-week-old human embryo was divided into four portions, namely, telencephalon (Tel), diencephalon (Di), mesencephalon (Mes), and rhombencephalon (Rhomb; metencephalon + myelencephalon). Each tissue was digested and dissociated into single cells and expanded in serum-free medium in the presence of FGF-2, EGF, and LIF. Within a few days, neurospheres started to form in all four cultures.

These cells were immunoreactive with Musashi-1 (Kaneko et al., 2000), which is one of the selective markers for neural stem cells (Fig. 1A). When the cells were cultured on polyornithine/laminin-coated dishes in the absence of growth factors, the expression of Musashi-1 decreased, and the number of MAP2ab-positive cells increased. With 14 days under this condition, each sphere extended long processes in a radial fashion. The cells within spheres moved along these processes and differentiated into MAP2ab-positive neurons and glial fibrillary acidic protein (GFAP)-positive astrocytes (Fig. 1B). Although they were very few, GalC-positive oligodendrocytes were also observed (Fig. 1C).

To evaluate the multipotency of these sphere-forming cells, clonal populations were prepared by the method of genomic selection. A gene encoding GFP and a hygromycin-resistant gene were introduced into the cells by using a retroviral vector, and single-cell-derived spheres were selected. Finally, four populations from telencephalon, four from diencephalon, three from mesencephalon, and one from rhombencephalon cultures were obtained. As a representative population, we used a population derived from the mesencephalon, which we named "M1." To confirm that the M1 cells were indeed a clonal population, we prepared genomic DNA from them and performed Southern blot analysis. If M1 cells were derived from a single cell, only one proviral band should be visible. As shown Figure 2A, only a single band was detected, which proved the clonality of these cells. Next, M1 cells were cultured for 14 days under the differentiating condition described above. A double-labeling immunofluorescence study showed that GFP-positive M1 cells also expressed Tuj1, a neuronal marker, or GFAP, an astrocytic marker, or O4, an oligodendroglial marker (Fig. 2B–D). These results demonstrate that the sphere-forming cells were multipotent NPCs.

Before starting this experiment, we cultured human NPCs from different part of the embryonic brains. In one case, the NPCs were isolated from telencephalon and

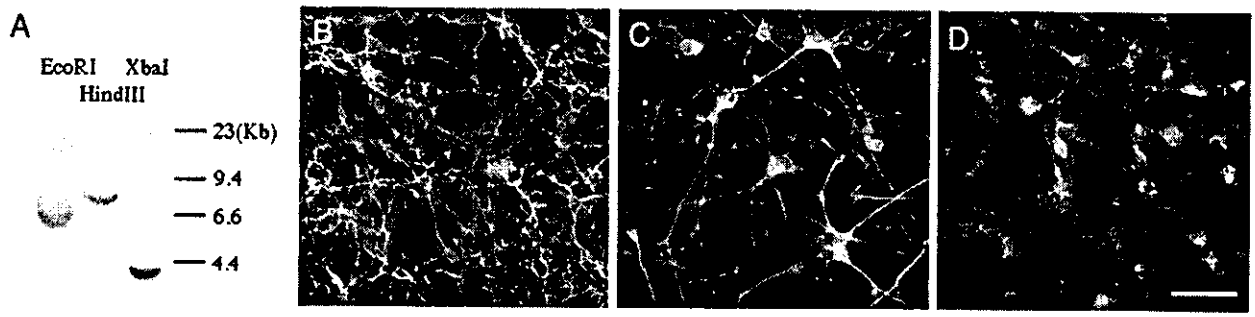

Fig. 2. Multipotency of human NPCs. **A:** Southern blot analysis of a representative population, M1. Single bands in each lane show that this population is monoclonal. **B–D:** Double-labeled confocal images of differentiated M1 cells. The cells expressed GFP (green) and gave rise to Tuj1-positive neurons (red in B), GFAP-positive astrocytes (red in C), and O4-positive oligodendrocytes (red in D). Scale bar = 50 μm.

mesencephalon/metencephalon of a 9-week-old embryo, and the former cells proliferated more rapidly than the latter cells. In another case, the NPCs were isolated from telencephalon and spinal cord of a 9-week-old embryo. However, the cells from the spinal cord ceased proliferating in 2 months, whereas the cells from telencephalon continued to proliferate with a doubling time of 8.6 days. In the present study, each precursor cell from four different regions of the brain continued to proliferate for over 1 year. However, when their proliferation rates were compared up to 100 days, there also was a tendency for the cells from the rostral part of the brain to proliferate faster than those from the caudal part (Fig. 3). The doubling times of each cell were 4.1 (Tel), 4.0 (Di), 4.4 (Mes), and 5.1 (Rhomb) days for the first 4 weeks and 7.7, 10.7, 12.6, and 17.4 days, respectively, for the last 4 weeks (Fig. 3).

As shown above, the NPCs proliferated as spheres with growth factors, and they began to differentiate when these factors were withdrawn. To compare the potential for giving rise to certain lineages among the NPCs of different origins, we cultured the cells as spheres for 2, 50, or 150 days and then caused them to differentiate. After 14 days of differentiation, immunofluorescence studies were performed. For neuronal subpopulations, anti-GABA antibody for GABAergic neurons and anti-TH antibody for dopaminergic neurons were used. As shown in Figure 4A, there were no significant differences among the origins of the cells for the percentage of MAP2ab-, GFAP-, or GABA-positive cells. The percentage of GFAP-positive cells dramatically increased as the culture period became longer, whereas that of MAP2ab- or GABA-positive cells remained unchanged or was slightly decreased. A significant difference among the origins of the cells was found with respect to TH-positive cells (Fig. 4B). The percentage of TH-positive cells was significantly higher with the precursors derived from the diencephalon and mesencephalon than with those from the telencephalon and rhombencephalon in 2-day- and 50-day-cultured spheres. A double-labeling immunofluorescence study of 50-day-cultured spheres also revealed a difference between origins (Fig. 5). In the cultures of the cells derived from the

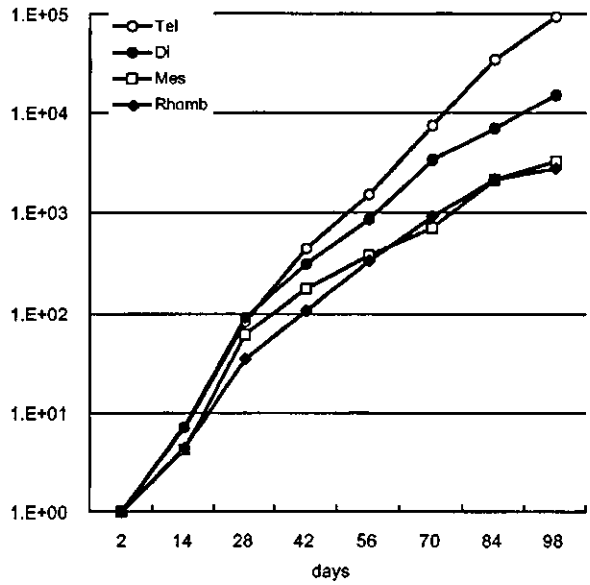

Fig. 3. Proliferation rate of human NPCs. Parallel cultures of NPCs derived from telencephalon (Tel), diencephalon (Di), mesencephalon (Mes), and rhombencephalon (Rho) were maintained in hFGF-2, hEGF, and hLIF. The ratios of total cell numbers to those on day 2 in each culture were plotted on a semilogarithmic graph.

telencephalon or rhombencephalon, all of the TH-positive cells were also GABA positive. Furthermore, these cells were small and bipolar in morphology. On the other hand, most of the TH-positive cells in the cultures of diencephalon- or mesencephalon-derived cells were GABA negative. Furthermore, these cells were larger than other cells and had long and multiple processes. In 150-day cultures, however, the percentages of TH-positive cells decreased to about 0.25% in each culture, and no significant differences were observed between origins

To investigate the mechanism underlying the origin-related differences in potential of NPCs giving rise to dopaminergic neurons, we examined the gene expression of region-specific transcription factors in 50-day cultures

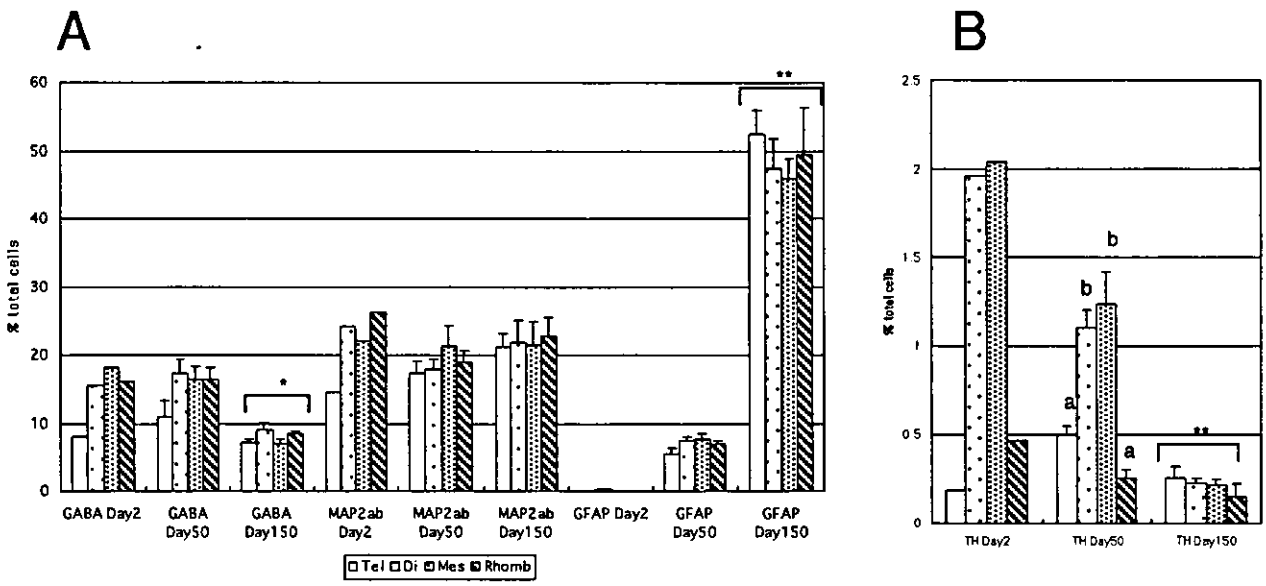

Fig. 4. Changes in the proportion of differentiated cells according to cell origin and culture period. **A:** Neurospheres derived from telencephalon (Tel), diencephalon (Di), mesencephalon (Mes), and rhombencephalon (Rhomb) were cultured for 2, 50, or 150 days and then made to differentiate for 14 days. The percentages of GABA-, MAP2ab-, and GFAP-positive cells per total cells are displayed as mean ± SEM of four to six independent experiments. Data of 2-day cultures, which were prepared in duplicate, are displayed without bars and statistical analysis. Statistical differences between 50- and 150-day cultures (*$P < .05$, **$P < .01$ vs. day 50), but none among origins, were found. **B:** Percentages of TH-positive cells. In 50-day cultures, neurospheres from Di and Mes gave rise to more TH-positive cells than those from Tel and Rhomb (values with different letters are significantly different at $P < .01$). In 150-day cultures, the percentages of each population decreased significantly (**$P < .01$) compared with those of 50-day cultures, but no significant differences were observed among origins.

of NPCs from each region. In the embryonic brain of rodents, Otx2 is expressed on the rostral side of the midbrain/hindbrain boundary, whereas Gbx2 is detected caudally (Joyner et al., 2000). This complementary pattern of expression in the anterior and posterior brain with a common border is important for normal patterning of the midbrain and cerebellum (Li and Joyner, 2001) and also for specification of dopaminergic and serotonergic neurons (Hynes and Rosenthal, 1999). Pax6 and Emx2 are expressed in the dorsal telencephalic neuroepithelium, which is the proliferative zone that gives rise to cortical neurons. Emx2 is has a low rostrolateral to high caudomedial gradient of expression, whereas Pax6 is expressed with this gradient reversed (Bishop et al., 2002). However, our RT-PCR study revealed that these genes were expressed in each precursor cell irrespective of their origin (Fig. 6A). For the differentiation of midbrain dopaminergic neurons, FGF8 and Shh are known to play an important role, so we next examined the expression of *Shh* and its receptors, *Ptc* and *Smo*, in NPCs from each region. Although *Shh* was not detected in any precursors (data not shown), both *Ptc* and *Smo* were expressed in all of them (Fig. 6B).

DISCUSSION

Previous reports showed that NPCs could be isolated from human embryonic brain (Svendsen et al., 1997; Vescovi et al., 1999; Carpenter et al., 1999; Ostenfeld et al., 2000). However, in most cases as well as in our previous cases, these cells were isolated from forebrain or some parts of the whole brain, probably because brains were often destroyed in the process of artificial abortion. In the present case, we were able to obtain an intact, whole brain from a 9-week-old human embryo, so we could dissect it precisely into four parts, namely, telencephalon, diencephalon, mesencephalon, and rhombencephalon (metencephalon + myelencephalon). This is important because it allowed us to compare the characteristics of NPCs derived from different part of the same brain. However, the results based on single brain might not be enough for us to reach broad conclusions. Detailed analysis on multiple intact brains will be required for that.

In each culture, the emerging neurospheres seemed similar. Because the cells constituting the neurospheres were nonadherent and freely floating, we thought that some form of genetic marking should be required for proving clonality, as was described in a previous report (Palmer et al., 1997). In the present study, for the first time, by using genetically proved clonal cells, we demonstrated that a human NPC was multipotent.

We observed two differences in NPCs in relation to their origin: 1) The NPCs from the rostral part of the brain tended to proliferate faster than those from the caudal part, and 2) the NPCs from the diencephalon and mesenceph-

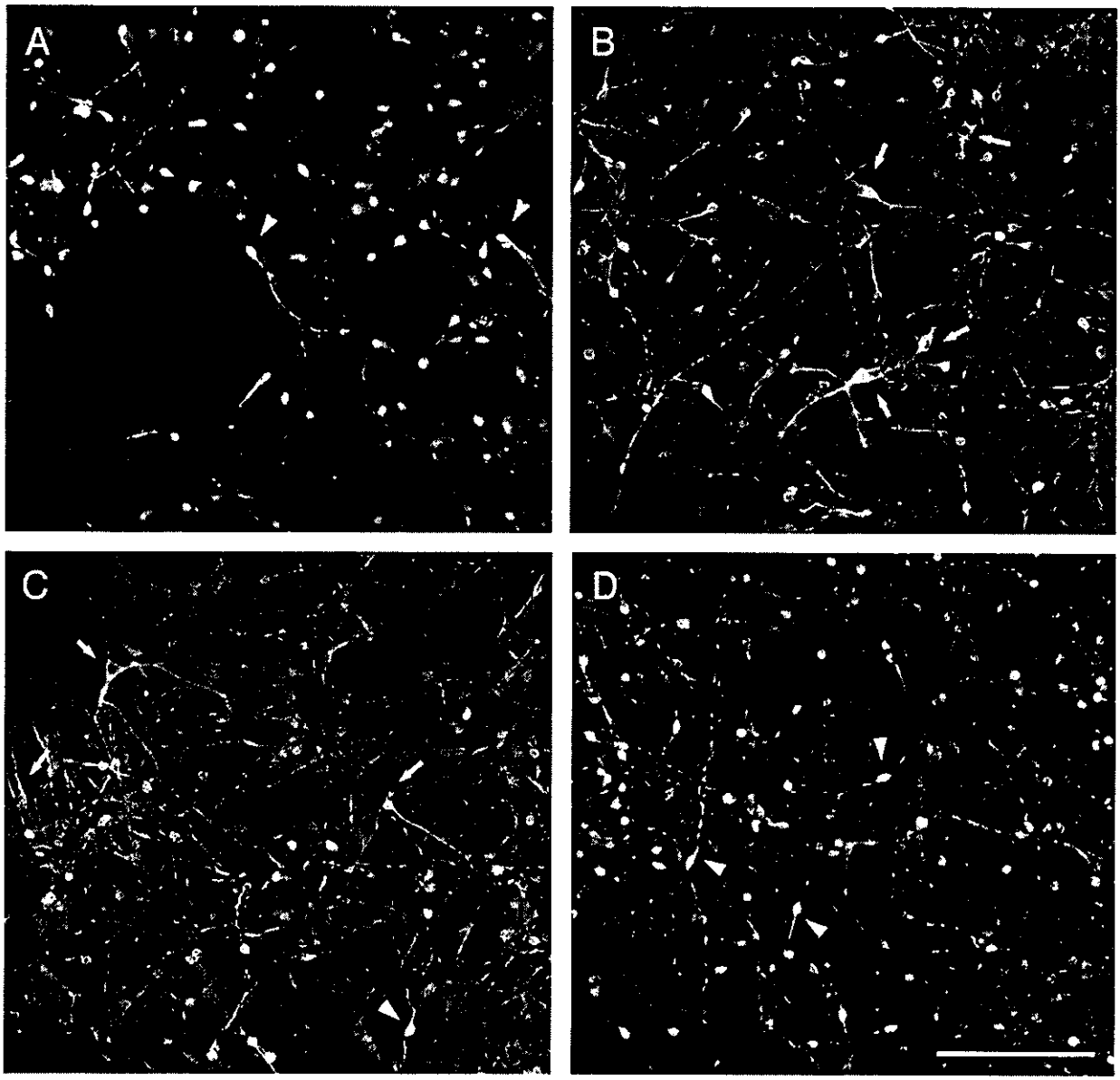

Fig. 5. Differentiation of TH- and GABA-positive neurons from each precursor. Double-labeling immunofluorescence staining of differentiated NPCs for TH (green) and GABA (red) was performed. Most of the TH-positive cells in the diencephalon (**B**) and mesencephalon (**C**) cultures were large, multipolar, and GABA negative (arrows). In contrast, TH-positive cells in the telencephalon (**A**) and rhombencephalon (**D**) cultures were small, bipolar, and GABA positive (yellowish orange, arrowheads). Scale bar = 100 μm.

alon gave rise to more TH-positive neurons than did those from the telencephalon and rhombencephalon. Furthermore, these TH-positive cells were different in morphology and GABA immunoreactivity. Recent evidence suggested that human NPCs from cortex proliferate faster than those from ventral mesencephalon and spinal cord in 35–70-day cultures (Jain et al., 2003; Ostenfeld et al., 2002). Our result obtained with 100-day cultures showing that NPCs from the rostral part, especially from the telencephalon, proliferated faster and longer is consistent with the earlier results. Also, this more rapid proliferation is reasonable in view of the facts that the telencephalon has a longer period of neurogenesis than the more caudal regions and that it forms the large cerebral cortex.

In our experiment on differentiation, a remarkable finding was the increase in the proportion of astrocytes in the long-term cultures. There were few astrocytes that had differentiated in fresh spheres, whereas about half of the cells were astrocytes in the case of 150-day-cultured spheres. This dramatic increase of GFAP-positive cells is

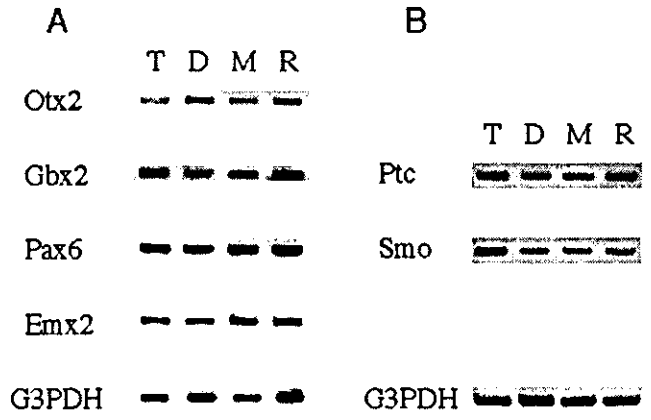

Fig. 6. Expression of region-specific transcription factors and Shh receptors. Total RNA from proliferating neurospheres on day 50 was analyzed. RT-PCR analysis demonstrated no distinct differences in expression of region-specific transcription factors (**A**) or Shh receptors (**B**) with respect to origins: telencephalon (T), diencephalon (D), mesencephalon (M), or rhombencephalon (R).

consistent with the facts that gliogenesis occurs after neurogenesis in normal development and that there are many more astrocytes in the brain. In contrast, the percentages of neurons and GABA-positive cells remained unchanged or decreased. The percentage of neurons after differentiation in long-term-cultured spheres was about 20% of the total cells and that of astrocytes 50%. This tendency is consistent with the previous findings (Carpenter et al., 1999; Jain et al., 2003). Because the percentage of oligodendrocytes is very low, MAP2ab- and GFAP-negative cells are presumed to be immature cells, such as neuronal or glial progenitor cells. On the other hand, it might be possible that these cells remain neural stem cells. Therefore, it is likely that these cells are immunoreactive against markers for more immature cells, such as Tuj1, MAP2c, A2B5, NG2.

With regard to TH-positive cells, there was a significant difference among the regions of cell origin, although there were no differences in this respect in the case of GABA-, MAP2ab-, and GFAP-positive cells. That is, more TH-positive cells were induced to differentiate from the diencephalic and mesencephalic precursors. It is possible that TH-positive cells from fresh spheres might include primary dopaminergic neurons. However, those from 50-day-cultured spheres had supposedly differentiated from neural stem cells, because it is unlikely for primary neurons to survive for such a long period without neurotrophic factors. As proved above, each sphere was derived from a multipotent precursor, so, if the spheres were completely triturated to a cell suspension for passage instead of being cut with microscissors, all of the TH-positive cells would be considered to be newborn neurons. These TH-positive cells were large, multipolar, and GABA negative and looked like primary midbrain dopaminergic neurons of rats (Ohmachi et al., 2003). Because of the limitation of cell numbers, no further analyses were performed. However, it is reasonable to assume that these large and multipolar cells were midbrain dopaminergic neurons. In contrast, TH-positive cells from telencephalic and rhombencephalic precursors were small, bipolar, and GABA positive. Virtually all the TH-positive periglomerular neurons in the rat olfactory bulb are also immunoreactive for GABA (Gall et al., 1987). There are newborn neurons immunoreactive for both TH and GABA in the olfactory bulb of newborn rats at postnatal day 2 (Betarbet et al., 1996), so it is possible that TH-positive cells from telencephalic precursors were such small neurons of the olfactory bulb. In agreement with our results, recent studies have demonstrated that human mesencephalic precursor cells gave rise to significantly more TH-positive neurons than telencephalic precursors in response to a cytokines/glial-derived neurotrophic factor (GDNF) mixture (Storch et al., 2001) or after transplantation into the rat brain (Sanchez-Pernaute et al., 2001).

Our RT-PCR study revealed that region-specific transcription factors, such as Otx2 and Gbx2, were expressed in proliferating human NPCs. Because this was not a quantitative RT-PCR, it is hard to discuss the difference in expression of these genes. However, there were, at least, no apparent differences in their expression with respect to their origin, which might help to explain the difference. It is known that the simultaneous presence of FGF8 and Shh is important for differentiation of progenitor cells into dopaminergic neurons (Ye et al., 1998). Our RT-PCR studies demonstrated that human NPCs expressed both *Ptc* and *Smo*, Shh-receptor genes. However, there were also no *apparent* differences in expression with respect to cell origin. A recent report suggests that NPCs are regionally specified as defined by expression of molecular markers, but this regional identity can be altered by local inductive cues (Hitoshi et al., 2002). It is possible that culture conditions may alter the expression patterns, which differed with respect to cell origin when the culture started. To discuss the difference and change in expression level of the genes, we have to perform quantitative RT-PCR or Northern blot analysis. However, we could not obtain enough cells to achieve it. These results suggest that human NPCs from each origin might have the genetic potential to differentiate into a variety of cells. The molecular mechanisms responsible for the regional specificity in proliferation rate and ability to give rise to dopaminergic neurons remain to be resolved. It will be necessary to perform the quantitative evaluations mentioned above, along with examination of other factors, such as Emx1, Dlx2, En1, Hoxb1, and Otx1.

Human NPCs are supposed to be a donor candidate for cell transplantation therapy for neurological disorders such as Parkinson's disease. Our present study demonstrated that multipotent NPCs can be expanded from the human embryonic brain and that the cells give rise to TH-positive neurons. However, because of regional specificity, NPCs derived from the mesencephalon are required to obtain midbrain dopaminergic neurons. Furthermore, the percentage of dopaminergic neurons drops

to 0.2% of total cells, whereas that of astrocytes increases up to 50% after a long-term culture. One of the advantages of using NPCs for cell transplantation is that they can be expanded almost unlimitedly. However, these results suggest that human NPCs expanded for a long period might have originated mainly from telencephalon and might give rise to few dopaminergic neurons and many astrocytes. For an effective cell transplantation therapy for Parkinson's disease, selective sorting and/or selective proliferation of dopaminergic progenitor cells will be necessary.

ACKNOWLEDGMENTS

We thank Hideyuki Okano for providing the antibody against Musashi-1 and Masato Nakafuku for technical advice on the RT-PCR study. We are grateful to Takuya Inoue, Hiroshi Hatayama, and Hirohiko Watanabe for the supply of human embryonic tissue. This work was supported by grants from the following programs: Grant-in-Aid for Scientific Research from the JSPS; Special Coordination Funds for Promoting Science and Technology from the MEXT; Health Sciences Research Grants in Research on Human Genome, Tissue Engineering, and Food Biotechnology from the MHLW of Japan.

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 Related Commentary, page 23

Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model

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Parkinson disease (PD) is a neurodegenerative disorder characterized by loss of midbrain dopaminergic (DA) neurons. ES cells are currently the most promising donor cell source for cell-replacement therapy in PD. We previously described a strong neuralizing activity present on the surface of stromal cells, named stromal cell–derived inducing activity (SDIA). In this study, we generated neurospheres composed of neural progenitors from monkey ES cells, which are capable of producing large numbers of DA neurons. We demonstrated that FGF20, preferentially expressed in the substantia nigra, acts synergistically with FGF2 to increase the number of DA neurons in ES cell–derived neurospheres. We also analyzed the effect of transplantation of DA neurons generated from monkey ES cells into 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine–treated (MPTP-treated) monkeys, a primate model for PD. Behavioral studies and functional imaging revealed that the transplanted cells functioned as DA neurons and attenuated MPTP-induced neurological symptoms.

Introduction

Parkinson disease (PD) is a neurodegenerative disorder characterized by the loss of midbrain dopaminergic (DA) neurons, with subsequent reductions in striatal dopamine levels. While initial pharmacological treatment with L-dihydroxyphenylalanin (L-DOPA) can attenuate symptoms, the efficacy of this treatment gradually decreases over time. The development of motor complications then requires additional treatments, including deep brain stimulation and fetal DA neuron transplantation (1–3). Both studies of animal models and clinical investigations have shown that transplantation of fetal DA neurons can produce symptomatic relief (4–8). The technical and ethical difficulties in obtaining sufficient and appropriate donor fetal brain tissue, however, have limited the application of this therapy.

ES cells are self-renewing, pluripotent cells derived from the inner cell mass of the preimplantation blastocyst. These cells have many of the characteristics required of a cell source for cell-replacement therapy, including proliferation and differentiation capacities (9). We previously discovered that a strong neuralizing activity, which we called stromal cell–derived inducing activity (SDIA), is present on the surface of stromal cells. In the absence of exogenous bone morphogenic protein–4, mouse ES cells differentiate efficiently into neural precursors and neurons when cultured for 1 week on SDIA-expressing mouse stromal cells (PA6 cells) (10). Recently, SDIA induction has also been applied to primate ES cells, which generated large numbers of neural precursors and postmitotic neurons when cultured on PA6 cells for two weeks (11). The SDIA method is both technically simple and efficient, producing significant numbers of midbrain DA neurons (10, 11).

Self-renewing, multipotent neural progenitors can be cultured as neurospheres (12). In this study, we generated neural progenitors from monkey ES cells, then expanded them as neurospheres, which contained progenitors of DA neurons. In addition, we analyzed the effect of FGF20, a novel member of the FGF family of growth factors that is expressed exclusively in the substantia nigra of the brain and is reported to have a protective effect on DA neurons (13). We observed increased DA neuron induction following treatment with FGF20. Furthermore, we transplanted neurons generated by this method into 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine–treated (MPTP-treated) cynomolgus monkeys, a primate model for PD. We found that transplanted cells were able to function as DA neurons and could diminish Parkinsonian symptoms. This is the first report, to our knowledge, demonstrating the efficacy of transplantation therapy using ES cell–derived DA neurons in an experimental primate model of PD.

Results

Induction of neural progenitors from monkey ES cells. To enrich graftable neural progenitors, we first cultured monkey ES cells on PA6 stromal feeder cells, then detached the cells from the feeders for

Nonstandard abbreviations used: ChAT, choline acetyltransferase; DA, dopaminergic; DAT, dopamine transporter; GABA, γ-amino butyric acid; GalC, galactocerebroside C; GFAP, glial fibrillary acidic protein; GMEM, Glasgow minimum essential medium; i.m., intramuscular(ly); LIF, leukemia inhibitory factor; ME, mercaptoethanol; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NCAM, N cell adhesion molecule; OL, poly-l-ornithine and laminin; PD, Parkinson disease; PET, positron emission tomography; SDIA, stromal cell–derived inducing activity; TH, tyrosine hydroxylase.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* **115**:102–109 (2005). doi:10.1172/JCI200521137.

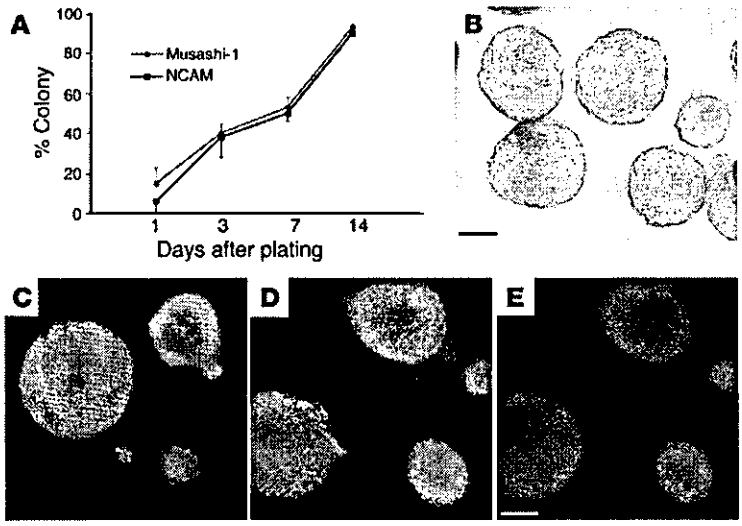

Figure 1
Neural progenitors induced from primate ES cells. (**A**) Time course of neural progenitor marker expression in monkey ES cells cultured on PA6 cells. (**B**) Detached ES cell colonies formed spheres similar to those of neural progenitor cells. (**C–E**) Spheres were immunoreactive for NCAM (**C**, green), Musashi-1 (**D**, red), and Nestin (**E**, green). Scale bar: 100 μm.

expansion as neurospheres. ES cells began to differentiate on PA6 cells, with cells immunoreactive for neural progenitor markers, such as N cell adhesion molecule (NCAM) and Musashi-1 (11, 14), emerging within three days. ES cells proliferated and differentiated on the feeder layer by forming colonies, and cells positive for neural markers increased in number until 2 weeks into the culture period. The percentages of the colonies including at least 1 NCAM- or Musashi-1-positive cell reached approximately 100% at 2 weeks of culture (NCAM = 90.4% ± 7.5%, Musashi-1 = 97.5% ± 4.6%, n = 100 from 3 independent cultures; Figure 1A). At this time point, 78.3% ± 7.5% of the total cells were immunoreactive for NCAM, 75.0% ± 15.4% for Musashi-1, and 72.3% ± 9.1% for another neural progenitor marker, Nestin (14). These results indicate that the majority of the ES cells were committed to the neural lineage by day 14 of culture on feeder layers.

In vitro characterization of neural progenitors derived from monkey ES cells. To further enrich neural progenitors, we detached the ES cells from the feeder layer on day 14, then continued to culture the cells on noncoated dishes in serum-free medium containing FGF2, EGF, and leukemia inhibitory factor (LIF). During the next few days, the cells formed spheres morphologically resembling those formed by neural progenitor cells (Figure 1B). These spheres were positively stained with antibodies specific for neural progenitor cell markers NCAM, Musashi-1, and Nestin (Figure 1, C-E). To determine the potential of these cells to differentiate, we expanded the cells as spheres for 7 days, then induced differentiation by culturing the cells on poly-l-ornithine and laminin-coated (OL-coated) slides for 7 days. We removed the mitogens FGF2, EGF, and LIF from the medium, instead adding neurotrophic factors such as brain-derived neurotrophic factor and neurotrophin-3. Immunofluorescence analysis revealed that the cells differentiated into mature neural cells expressing the neuronal markers TuJ1 (52.8% ± 16.0% of DAPI) and Map2ab (38.3% ± 7.5% of DAPI), the astroglial marker glial fibrillary acidic protein (GFAP) (28.6% ± 17.6% of DAPI), and the oligodendroglial marker galactocerebroside C (GalC) (0.6% ± 0.4% of DAPI) (Figure 2, A-D and I). Further analyses demonstrated that these neurons derived from ES cells were immunoreactive for γ-amino butyric acid (GABA) (28.6% ± 10.7% of TuJ1), glutamate (14.3% ± 5.3% of TuJ1), choline acetyltransferase (ChAT) (0.7% ± 0.3% of TuJ1), and serotonin (3.3% ± 1.7% of TuJ1) (Figure 2, E-H and J). These results suggest that ES cell-derived spheres contain neural progenitor cells.

Effect of growth factors on differentiation of DA neurons. Effective treatment of PD requires substantial quantities of DA neurons. The percentage of tyrosine hydroxylase-positive (TH-positive) cells derived from neurospheres was only 5.4% ± 1.8% of the TuJ1-positive cells (Figure 2J). To increase the percentage of these cells, we examined the effects of various combinations of growth factors on neurosphere culture. The percentage of

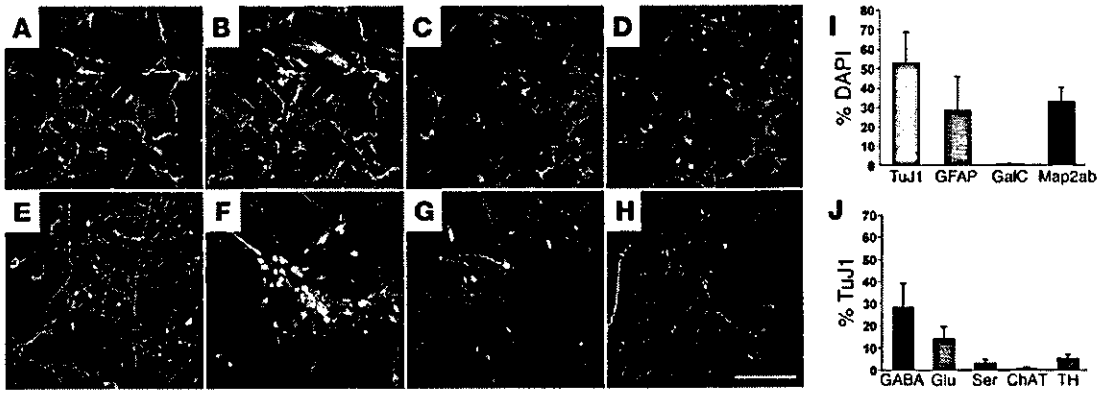

Figure 2
Expression of differentiated neural and neuronal subtype markers. Differentiated spheres were stained with antibodies against TuJ1 (**A** and **B**, green; **E–H**, blue), GFAP (**B**, red), Map2ab (**C** and **D**, green), GalC (**D**, red), GABA (**E**, red), glutamate (Glu; **F**, green), serotonin (Ser; **G**, red), and ChAT (**H**, green). Scale bar: 100 μm. The proportions of cells expressing differentiated neural (**I**) and neurotransmitter-related (**J**) markers are expressed as the mean ± SD of 3 independent cultures.