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Neural precursor cells from adult mouse cerebral cortex differentiate into both neurons and oligodendrocytes

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Abstract

Recent findings concerning adult neurogenesis in two selected structures of the mammalian brain, the olfactory bulb and dentate gyrus of the hippocampus, present the possibility that this mechanism of neurogenesis applies for all brain regions, including the cerebral neocortex. In this way, a small number of potential neural precursor cells may exist in the cerebral neocortex, but they do not normally differentiate into cortical neurons *in vivo*. It has, however, been reported recently that cycling cells isolated from non-neurogenic areas of adult rat cerebral cortex could generate neurons *in vitro*. In this study, we analyzed the lineage potential of cycling cells from the adult mouse neocortex. For the dissection of the cerebral cortex from the adult mouse, which is significantly smaller than that of the adult rat, we have modified the previous dissection protocol developed for the rat neocortex. As a result, cycling cells from adult mouse neocortex gave rise to neurons and oligodendrocytes, but not to astrocytes, whereas when the previous dissection method was used, cycling cells gave rise to neurons, oligodendrocytes and astrocytes. This discrepancy might stem from slight contamination of the dissected mouse neocortical tissue in the previous protocol used for the dissection of rat neocortex by cells from the surrounding subependymal zone, where typical adult neural stem cells exist. The results presented here will contribute to our understanding of the nature of cycling cells in the adult mammalian neocortex, and for which future stem cell research will provide new possibilities for cell replacement therapy to be used in the treatment of neurodegenerative conditions.

Abbreviations: EGF – epidermal growth factor; FGF – fibroblast growth factor; GFAP – glial fibrillary acidic protein; HBSS – Hank's buffered saline solution; MAP-2 – microtubule associated protein-2; SVZ – subventricular zone

Introduction

Cell proliferation in the adult mammalian brain is ubiquitous, but neurogenesis occurs only in two specific brain regions: the olfactory bulb and the dentate gyrus of the hippocampus (Gage 2000; Rakic 2002). In the adult mammalian neocortex, it has been proposed that cycling cells belong to glial lineages (Levison et al. 1999; Gensert and Goldman 2001). However, it was recently reported that

precursors native to regions of the adult brain that generate only glia could generate neurons *in vitro* (Palmer et al. 1999; Kondo and Raff 2000). In particular, Palmer et al. (1999) reported on the nature of cycling cells isolated from the adult rat CNS. They demonstrated that cycling cells isolated from adult rat cortex could give rise to neurons only after exposure to FGF-2. In addition, cycling cells isolated from non-neurogenic areas in adult brain could give rise to neurons after grafting back to neurogenic regions *in vivo* (Shihabuddin et al. 2000). On the basis of a latent neurogenic program in neural stem cells being activated, it is possible that cells from non-neurogenic regions of the adult central nervous system also give rise to neurons.

In the subventricular zone (SVZ) of the adult mammalian brain, neural stem cells give rise to new neurons. Cells isolated from the SVZ can thus be cultured in the presence of mitogens such as fibroblast growth factor (FGF), epidermal growth factor (EGF), or the two combined (Weiss et al. 1996; Temple and Alvarez-Buylla 1999; Gage 2000) and can be induced to differentiate by withdrawing the mitogens or by exposing the cells to another factor such as bone morphology protein (Lim et al. 2000). SVZ neural stem cells exist *in vivo* in the subependyma surrounding the lateral ventricles, a region which is in close proximity to the neocortex. To analyze the properties of neocortical precursor cells, the cerebral cortex must be carefully dissected free of neighboring white matter, which may include precursor cells from the SVZ. In this study, in order to analyze the properties of cycling cells from the adult mouse neocortex, we modified the dissection protocol used by Palmer et al. (1999) for isolation the rat neocortex.

Recently, attempts have been made to use neural stem cells for the repair of neurologically diseased areas of the CNS (Bjorklund and Lindvall 2000). Transplantation of fetal mesencephalic cells into patients with Parkinson's disease (Bjorklund and Lindvall 2000) and transplantation of fetal striatum into patients with Huntington's disease (Bachoud-Levi et al. 2000) have already been carried out and led to symptomatic improvement. However, these experiments have problems associated with ethical constraints, viability and purity of the cells and the absence of cell proliferation. Stem cell research will thus provide new possibilities for cell replacement therapy to treat neurological diseases. In this study, we focused on the properties of cycling cells isolated from the neocortex of adult mice.

Materials and methods

Tissue dissection

Adult male and female ICR mice (8 weeks) were purchased from Sankyo Lab Service (Tokyo, Japan). All experiments were carried out in accordance with the guidelines for Animal Experiments of the Faculty of Frontier sciences, The University of Tokyo. The neocortex and the SVZ were dissected and the resultant tissue finely minced and digested in a solution of 2.5 U ml^{-1} papain (Worthington, New Jersey, USA), 250 U ml^{-1} DNase (Worthington), and 1 U ml^{-1} Dispase (Boehringer Mannheim, Baden Wurttemberg, German) dissolved in Hank's balanced saline solution (HBSS). Cells and tissue fragments were washed three times with DMEM/F12 (1:1) (GIBCO BRL, Maryland USA) containing 10% fetal bovine serum (FBS) (GIBCO BRL). Whole digested tissue was then suspended in DMEM/F12 containing 10% FBS, filtered through a sterile 70 μm nylon mesh and thoroughly mixed with an equal volume of percoll solution. The percoll solution was made by mixing nine parts of Percoll (Amersham Pharmacia Biotech, New Jersey, USA) with one part 10 $\times$ PBS. The cell suspension was then fractionated by centrifugation at 20,000*g* for 30 min at 18 °C. Colored beads of known buoyancy (Amersham Pharmacia Biotech) were used to calibrate the cell gradient. Cell fractions were harvested and washed with DMEM supplemented with 10% FBS as described by Palmer et al. (1999).

Cell culture

Primary cells from the neocortex or the SVZ of adult mouse were plated on poly-ornithine/fibronectin/laminin-coated dishes in DMEM/F12 containing 10% FBS and maintained in this manner for 24 h. The medium was then replaced with DMEM/F12 containing FGF-2 (20 ng ml^{-1}) and EGF(20 ng ml^{-1}) (growth medium). Seventy-five percent of the medium was replaced with new growth medium every 48 h. To promote

differentiation, the growth medium was replaced with DMEM/F12 supplemented with N_2supplement. DMEM/F12 containing 10% FBS, FGF-2 and EGF was used to favor astrocyte differentiation.

Immunostaining

Cells were fixed with 4% paraformaldehyde in PBS for 20 min. After incubation with a blocking solution (3% NGS and 0.1% Triton X-100 in PBS or 3% NGS in PBS) for 2 h at room temperature, cells were incubated overnight with primary antibodies at 4 °C. Primary antibody concentrations used are as follows: Anti-microtubule associated protein-2 (MAP-2), 1:500 (Sigma), anti-glial fibrillary acidic protein (GFAP), 1:10000 (DAKO, Glostrup, Denmark), anti-O1, 1:100 (CHEMICON, CA, USA). Cells were then incubated for 2 h with a secondary antibody (Yamada et al. 1999). Secondary antibody concentrations used are as follows: Donkey anti-mouse IgG rodamin, 1:200 (ICN, CA, USA), donkey anti-rabbit IgG alexa488, 1:500 (Molecular Probes), goat anti-mouse IgM AMCA, 1:200 (CHEMICON). After washing, the cells were mounted in DABCO/immunoblot. Labeled cells were visualized using confocal scanning laser microscopy (Leica, Mannheim, Germany).

Results

Development of a new dissection protocol for isolation of the murine cerebral neocortex

In this study, to analyze cells isolated from the neocortex we developed a modified tissue dissection protocol. Since the mouse brain is smaller than the rat brain, the precise micro-dissection of cerebral cortex, which must be absolutely free of surrounding white matter, is therefore particularly difficult if realized using the previous protocol developed for rat neocortex by Palmer et al. (1999). In brief, the brain was removed and placed in HBSS. Observed with the aid of a dissecting microscope, the brain was cut using a scalpel into a coronal section of about 2 mm thickness, which did not include the frontal or occipital lobes (Figure 1a). The surrounding white matter can be clearly visualized when the section is examined with the aid of the microscope (Figure 1b), and the neocortex can therefore be easily dissected along the boundary of the white matter using regular fine dissecting forceps. Contamination by the white

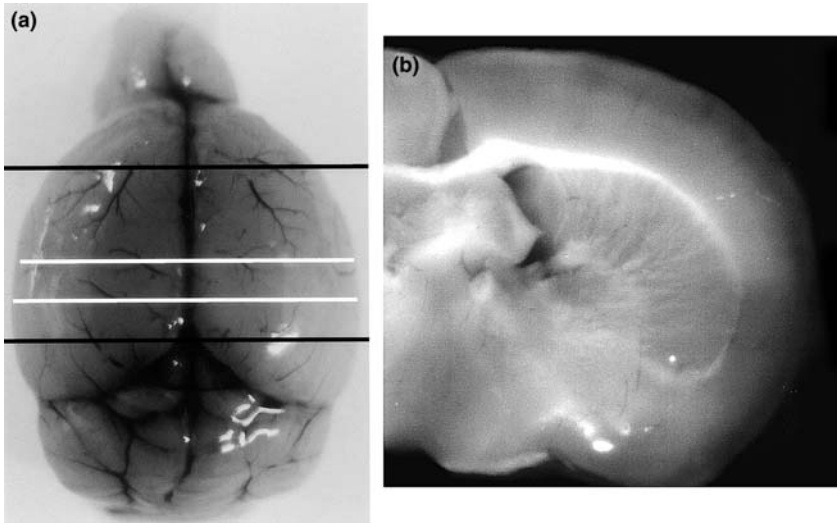

Figure 1. Dissection of cerebral cortex from adult mouse brain. (a) Tissues used for dissection are located between overlying black lines. Brain was first cut using a scalpel into a coronal section of about 2mm thickness (white lines). (b) In a cortical brain slice, the boundary between the neocortex and white matter is clearly apparent. Using forceps, the neocortex (highlighted by white line) was carefully separated from the rest of the brain slice.

matter, where typical adult neural stem cells are likely to have migrated from the subependymal zone of the lateral ventricle, can thus be completely avoided. Throughout the dissecting procedure, the HBSS was bubbled with a 95%/5% O_2/CO_2 gas mixture to prevent the possible cell damage due to a lack of oxygen. Cycling cells were then condensed by a protocol based on buoyant density (Palmer et al. 1999) and cultured with defined growth medium.

Analysis of lineage potential

Neocortical cycling cells cultured in serum-free growth medium are able to generate several colonies of cells (Figure 2a). To determine the lineage potential of cycling cells from the neocortex, growth medium was replaced with differentiation medium (1 ng ml^{-1} bFGF, 1% FCS, and 100 ng/ml all-trans retinoic acid) (Palmer et al. 1997; Takahashi et al. 1999) and these cells were allowed to differentiate for 6 days. Neocortical cycling cells gave rise to neurons (MAP-2; Figure 2b) and oligodendrocytes (O1; Figure 2c), but not to astrocytes (data not shown).

To compare the results obtained using our modified dissection protocol with results obtained using the previous protocol described by Palmer et al. (1999), cells were also prepared using the method. Cycling cells were cultured with growth medium and differentiated for 6 days. As shown in Figure 3, cells differentiated into neurons (Figure 3a), astrocytes (Figure 3b), and oligodendrocytes (Figure 3c), indicating a different potential for differentiation of cycling cells realized by the previous protocol, which probably contains progenitor cells from white matter. Recently, Nunes et al. (2003) have succeeded in isolating multipotential neural progenitor cells from the subcortical white matter from the adult human brain. As a control experiment, cycling cells isolated from the adult mouse SVZ were also cultured in a similar manner to the above, and gave rise to neurons, astrocytes and oligodendrocytes (Figure 3d and e). Taken together, it can be assumed that the nature of cycling cells obtained from the adult neocortical region using the Palmer et al. dissection protocol is consistent with that of the adult SVZ.

Cycling cells isolated solely from the adult neocortex do not generate astrocytes

To assess whether proliferating cells in the adult neocortex are capable of generating astrocytes or not, neocortical cycling cells were cultured in serum-supplemented medium, which promotes extensive astrocytic differentiation (Gensert and Goldman 2001). When cycling cells prepared by the modified dissection protocol described here were cultured in serum-supplemented medium, several colonies of cell types were generated. According to identification by immunocytochemistry, some cycling cells differentiated into neurons (Figure 4a) and many cells into oligodendrocytes (Figure 4b), none differentiated into GFAP-positive astrocytes. In a control experiment, cycling cells from the SVZ were also cultured under the same medium conditions; these proliferated and were induced to differentiate as described above. Immunocytochemistry results showed that these cells gave rise to neurons, oligodendrocytes and many astrocytes (Figure 4c and d).

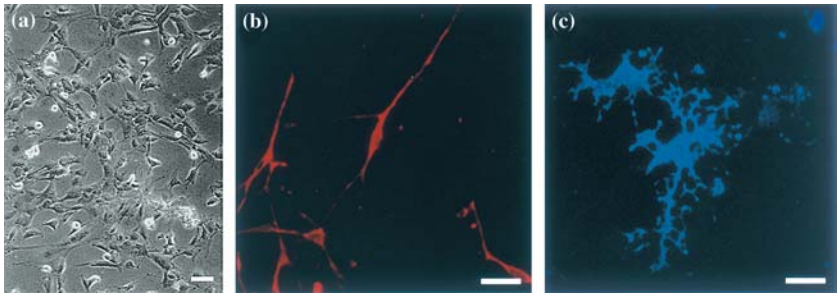

Figure 2. Neurogenesis in a culture of cells obtained from a precisely isolated neocortex from adult mouse brain. (a) A typical cell population cultured in growth medium. Cells in the differentiation medium were immunoreactive for MAP-2 (b) and O1(c). Similar results were obtained from several separate experiments. Scale bars, 20 μm.

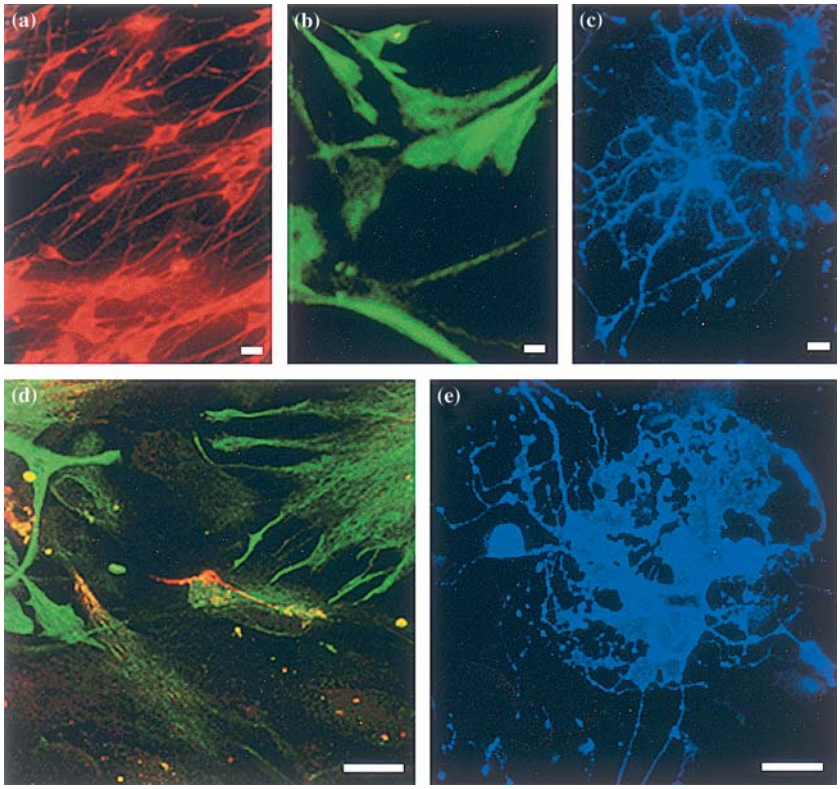

Figure 3. Lineage potential of cells isolated from mouse neocortex using the protocol of Palmer et al. and of cells from the SVZ. Cells isolated from the neocortex by the previous protocol differentiated into neurons and glia. MAP-2-positive neurons are shown in red (a), GFAP-positive astrocytes in green (b), and O1-positive oligodendrocytes in blue (c). Similarly, SVZ cells differentiated into neurons and glia. MAP-2-positive neurons are shown in red, GFAP-positive astrocytes in green (d), and O1-positive oligodendrocytes in blue (e). Similar results were obtained from several separate experiments. Scale bars, 20 μm.

Discussion

In this study, we have modified a previously reported dissection protocol for isolating cycling cells from the rat neocortex, and compared the two protocols in relation to the isolation of cycling cells from the mouse neocortex. The nature of the cell populations isolated from the adult mouse neocortex by our modified dissection protocol differed from what was obtained with the previously described protocol. A possible reason for this anomaly could be that, for the procedures used in the previous dissection protocol, the murine neocortex was contaminated with surrounding brain areas, such as white matter or tissue from the subependymal zone. Because the mouse brain is quite small in comparison to the rat brain, it is particularly difficult to accurately dissect out only the cerebral neocortex. It is also possible that the differentiation potential of mouse neural progenitors would not be comparable to that of rat neural progenitors. Under our culture conditions, cells isolated from the mouse neocortex differentiate into both neurons and oligodendrocytes, but not into GFAP-positive astrocytes. Previous study has reported that similar bipotential cells with the ability to generate both neurons and oligodendrocytes exist in the embryonic rat cerebral cortex (Williams et al. 1991). Because we did not detected astrocytes in our culture condition, these bipotential cells might exist in adult mouse cerebral cortex. In addition, recent study reported that glial progenitor cells from the subcortical white matter of adult human brain could generate neuron and glia both *in vitro* and *in vivo* (Nunes et al. 2003). These glial progenitor cells give rise to multipotent neurospheres and these spheres differentiate neuron and glia. In our study, however, the frequency of neuronal differentiation was extremely low (<1%/total cells),

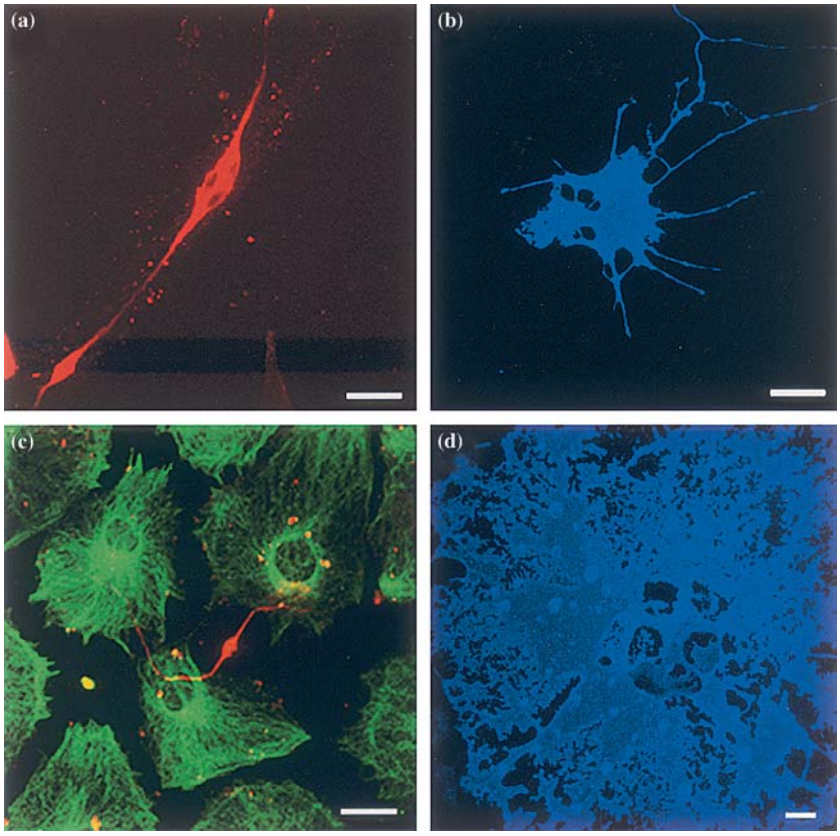

Figure 4. Cells isolated from the neocortex using our modified protocol did not generate into GFAP-positive astrocytes. Cells were cultured in serum-supplemented medium to promote astrocyte differentiation and induced differentiation. Cells isolated specifically from the neocortex gave rise to neurons and oligodendrocytes. MAP-2-positive neurons are shown in red (a) and O1-positive oligodendrocytes in blue (b). SVZ cells differentiated into neurons and glia. MAP-2-positive neurons are shown in red, GFAP-positive astrocytes in green (c), and O1-positive oligodendrocytes in blue (d). Scale bars, 20 μm.

probably because of our culture conditions. Our dissection protocol is effective in preventing contamination of the murine neocortex with white matter cells or SVZ neural stem cells.

Cell characteristics are often quite different in adjacent brain areas (Chiasson et al. 1999; Seaberg and van der Kooy 2002). For example, adult forebrain subependymal cells possess neural stem cell characteristics, but ependymal cells have only proliferative potential. Therefore, exact anatomical techniques are required for the dissection of brain tissue from each animal species. Among the experimental mammals, the mouse is the most commonly used. Now, with many mutated mouse strains having been developed by genetic engineering, provision has been made for animal models to be used in the treatment of animal models for human neurological diseases. In effect, transgenic mice for the treatment of Alzheimer's disease and Huntington's disease have already been established (Yu and Oberto 2000; Dedeoglu et al. 2002).

The cerebral neocortex is the center for human intelligence, and this area of the brain is often affected in neurodegenerative diseases such as Alzheimer's disease. Therefore, we analyzed the lineage potential of neocortical cycling cells, which, in the adult rat neocortex, have been reported to preferentially generate oligodendroglia (Levison et al. 1999). In this study, we also observed the extensive generation of O1-positive oligodendrocytes from cycling cells. Regarding astrocytic differentiation, the cells isolated from the adult mouse neocortex did not generate GFAP positive astrocytes. However, cells isolated from the adult mouse SVZ differentiated into astrocytes, thereby demonstrating that it is likely that the nature of cycling cells in the adult mouse neocortex is different from

that in SVZ. Most importantly, our results clearly demonstrate the neuronal differentiation of cycling cells from the cerebral neocortex, a non-neurogenic region of the adult CNS. Our results provide new possibilities for cell replacement therapy in the treatment of neurological diseases with associated cortical damage.

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