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Differing *in vitro* Survival Dependency of Mouse and Rat NG2⁺ Oligodendroglial Progenitor Cells

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Abstract

NG2 chondroitin sulfate proteoglycan is a surface marker of oligodendroglial progenitor cells (OPCs) in various species. In contrast to well-studied rat OPCs, however, we found that purified mouse NG2 surface positive cells (NG2⁺ cells) require additional activation of cyclic AMP (cAMP) signaling for survival in a medium containing 30% B104 neuroblastoma conditioned medium supplemented with fibroblast growth factor-2 (B104CM+FGF2), whereas B104CM +FGF2 alone is sufficient for survival and selective proliferation of rat OPCs. After induction of *in vitro* differentiation, more than 90% of mouse NG2⁺ cells became O4-positive, and a majority expressed myelin basic protein by 5 day of differentiation, which confirmed the identity of isolated mouse NG2⁺ cells as OPCs. In comparison to rat OPCs, mouse OPCs in B104CM+FGF2 were less motile, and demonstrated lower basal phosphorylation levels of ERK1/2 and cAMP response element-binding protein (CREB) and a higher incidence of apoptosis mediated by the intrinsic pathway. Transient up-regulation of cAMP-CREB signaling partially inhibited apoptosis of mouse OPCs independently of the ERK pathway. This study demonstrates a difference in trophic requirements between mouse and rat OPCs, with an essential role for cAMP signaling to preserve viability of mouse OPCs.

Keywords

mouse oligodendroglial progenitor cell; NG2 chondroitine sulfate proteoglycan; cyclic AMP signaling; cyclic AMP response element-binding protein; extracellular signal-regulated kinase

Introduction

Oligodendroglial progenitor cells (OPCs), derived either from donor tissues or from pluripotent stem cells, are the best candidate for transplantation therapies to enhance CNS remyelination after genetic, inflammatory, chemical or traumatic demyelination (Reviewed by Pluchino et al., 2004; Goldman, 2005). In support of this, extensive remyelination by transplanted OPCs from rats (Pluchino et al., 2003) and humans (Windrem et al., 2004) has been demonstrated in rodent models of CNS dysmyelination or demyelination, whereas more differentiated oligodendroglial lineage cells, such as O4 positive (Warrington et al.,

1993) or mitotically inactive cells (Crang et al., 1998) were less capable than OPCs of remyelination after transplantation. As a practical issue, OPCs for transplantation will need to be purified, accumulated and selectively expanded in order to produce sufficient numbers for therapeutic application.

Our current knowledge of OPCs is largely derived from studies of rat OPCs, particularly those historically called O-2A progenitor cells because of their capacity to differentiate into either oligodendrocytes or type II astrocytes *in vitro* (Raff et al., 1983). In the rat, pure OPCs can be isolated with the aid of A2B5 monoclonal antibody (Barres et al., 1992; Robinson and Miller 1996), and expanded *in vitro* for more than a year in the presence of platelet-derived growth factor A homodimer (PDGF_{AA}) and fibroblast growth factor 2 (FGF2), under which conditions their intrinsic maturation program (Bögler and Noble, 1994; Tang et al., 2000) and replicative cell senescence (Tang et al., 2001) are inhibited. In contrast to the rat, although several studies using mouse oligodendroglial cultures have been reported (Suzumura et al., 1984; Barres et al., 1996; Ghiani et al., 1999; Billon et al., 2001; Seiwa et al., 2002), it is still technically challenging to purify and expand numbers of pure mouse OPCs *in vitro* (Vitry et al., 1999). This difference between these two rodent species has not been explained. Some studies have pointed out that A2B5 antigen is not a good surface marker of mouse OPCs, because mouse OPCs express less A2B5 antigen than rat OPCs (Fanarraga et al., 1995; Duchala et al., 1995; Barres et al., 1996). On the other hand, NG2 chondroitin sulfate proteoglycan (NG2 CSPG) is surface expressed on the OPCs of both rodent species *in vivo* (Reviewed by Nishiyama et al., 2009; Polito and Reynolds 2005), and has been shown to be useful to isolate mouse OPCs (Diers-Fenger et al. 2001).

Since Bottenstein and her colleagues (1988) discovered preferential proliferation of rat OPCs in B104 neuroblastoma conditioned medium (B104CM), B104CM has been widely used not only for primary rat OPC cultures (Horiuchi et al., 2006; Itoh et al., 2002) and rat OPC lines such as CG4 (Louis et al., 1992), but also for preparation of mouse OPCs from multipotent neural progenitor cells (Vitry et al., 1999; Chen et al., 2007). Subsequent studies confirmed that B104CM contains PDGF_{AA} and neuregulin 1, both of which promote proliferation of OPCs (Asakura et al., 1997; Canoll et al., 1996). We initially expected that mouse OPCs would survive and proliferate in B104CM supplemented with FGF2, because the presence of both PDGF_{AA} and FGF2 in the defined culture medium has been shown to be sufficient for rat OPCs to survive and proliferate (Tang et al., 2000). However, our present study clearly demonstrates that mouse OPCs cannot survive in B104CM supplemented with FGF2 in the absence of additional cyclic AMP signaling. Thus, there is a significant qualitative difference in OPCs between rats and mice, suggesting that knowledge obtained from rat OPCs cannot safely be generalized to other species.

Materials and methods

Reagents and chemicals

All reagents and culture media used in this study were purchased from SIGMA (St. Louis, MO) and Invitrogen (Carlsbad, CA), respectively, except for the following products. Human recombinant FGF2 and PDGF_{AA} were from R&D systems (Minneapolis, MN); rabbit anti-ERK1/2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-phospho-ERK1/2 (catalog number 9101), rabbit anti-phospho-cAMP response element-binding protein (CREB) (catalog number 9198), rabbit anti-phospho-AKT (catalog number 9271), and rabbit anti-AKT antibodies were from Cell Signaling Technology (Danvers, MA); rabbit anti-NG2 chondroitin proteoglycan (catalog number AB5320), mouse anti- β -III tubulin, and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were from Millipore (Temecula, CA). Rat anti-myelin basic protein (MBP) antibody was

from Novus biological (Littleton, CO). Mouse anti-CD11b antibody was from Serotec (Raleigh, NC).

Cell culture

Mixed glial culture—Primary mixed glial cultures from mice and rats were prepared as reported previously (Itoh et al., 2002). Briefly, whole brains were dissected from 0 to 2-day-old (PN0-PN2) C57BL/6 mice and Lewis rats, and submerged in ice-cold Leibovitz's L-15 medium. Under a dissecting microscope, olfactory bulbs, cerebral cortexes and hindbrains were removed. After cleaning off meninges and vessels including choroidal plexus, the remaining brain tissues were cut into small chunks with a 21-gage needle, and digested by 0.0625% (w/v) trypsin in Ca^{2+} and Mg^{2+} -free Hank's Balanced Salt Solution (HBSS) for 20 min. Dissociated cells were obtained by passing the softened chunks through a 1 ml hand pipette tip several times, and collected by centrifugation at $365 \times g$ for 5 min. The cells were resuspended in minimum essential medium alpha containing 5% (v/v) fetal bovine serum and 5% (v/v) calf serum, and plated onto a 10 cm culture dish. One day after plating, attached cells (designated as passage 0) were washed with HBSS to remove serum, and thereafter maintained in the medium (GM), a 3:7 mixture (v/v) of B104 neuroblastoma-conditioned medium and the N1 medium (high glucose Dulbecco's modified Eagle's medium supplemented with 6 mM L-glutamine, 10 ng/ml biotin, 5 $\mu\text{g}/\text{ml}$ insulin, 50 $\mu\text{g}/\text{ml}$ apo-transferrin, 30 nM sodium selenite, 20 nM progesterone and 100 μM putrescine as final concentrations). Cultures were fed with fresh GM every other day for approximately 5 days, at which time the proliferating glial cells were almost confluent.

Isolation of mouse and rat NG2⁺ cells by flow-based cell sorting—Mixed glial cells in a 10 cm dish were washed with 5 ml Ca^{2+} and Mg^{2+} -free HBSS, and then 1 ml trypsin-EDTA (0.05% (w/v) and 0.53 mM, respectively, in Ca^{2+} and Mg^{2+} -free HBSS) was added. After incubation at room temperature for 30 sec, trypsin was immediately inactivated by adding 5 ml GM containing 0.625 mg/ml trypsin inhibitor, and detached cells were collected to a 15 ml tube and centrifuged at $400 \times g$ for 5 min. This trypsinization method is milder and shorter than general trypsinization to retain antigenicity of the NG2 molecules as much as possible, because NG2 CSPG is known to be cleaved by trypsin (Nishiyama et al., 1995). The collected cells were resuspended in 500 μl of 0.1% fraction V bovine serum albumin in phosphate buffered saline (0.1% BSA/PBS) containing 5 μl of rabbit anti-NG2 antibody and incubated on ice for 30 min. After washing 3 times with 1 ml 0.1% BSA/PBS, cells were resuspended in 0.1% BSA/PBS with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:50) and incubated on ice for 40 min. Then cells were washed 3 times again and resuspended with 400 μl of 0.1% BSA/PBS with 1 $\mu\text{g}/\text{ml}$ propidium iodide (PI) for cell sorting. FITC⁺/PI⁻ cells were isolated by MoFlo, a flow-based cell sorter (Cytomation, Fort Collins, CO), as described in Results.

Immunopanning for purification of A2B5⁺ rat OPCs—Some rat OPCs were obtained by the well-established A2B5 immunopanning method (Itoh et al., 2002). Briefly, rat mixed glial cultures were washed with Ca^{2+} and Mg^{2+} -free HBSS, suspended in the N1 medium containing 0.1% (w/v) BSA, and plated and incubated on the negative immunopanning plates coated with RAN-2 antibody for 30 min at 37 °C to exclude RAN-2-positive population (Itoh et al., 2002). Following two rounds of this negative selection, nonadherent cells were transferred to the A2B5 positive panning plates. After the serial immunopanning, purified cultures contained more than 95% of A2B5-positive OPCs.

***In vitro* differentiation of mouse and rat OPCs**—To induce *in vitro* differentiation of OPCs, the culture medium was switched from the GM to differentiation medium (DM), a 1:1 mixture (v/v) of high glucose Dulbecco's modified Eagle's medium and Ham's F-12

medium supplemented with 4.5 mM L-glutamine, 10 ng/ml biotin, 12.5 µg/ml insulin, 50 µg/ml transferrin, 24 nM sodium selenite, 10 nM progesterone, 67 µM putrescine, 0.4 µg/ml 3,5,3',5'-tetraiodothyronine, 100 U/ml penicillin and 100 µg/ml streptomycin as final concentrations.

Immunocytochemistry

Cells cultured on poly-D-lysine-coated coverslips were incubated with primary antibodies against cell surface antigens, such as hybridoma supernatants of A2B5, O4 and H8H9 (no dilution), and anti-NG2 (1:50) and anti-CD11b antibodies (1:50), at room temperature for 30 min. After washing with PBS, cells were fixed with 4% paraformaldehyde at room temperature for 15 min, and then permeabilized with 100% methanol at -20°C for 15 min. Incubation with antibodies against intracellular molecules, such as anti-MBP (1:2), glial fibrillary acidic protein (GFAP) (1:200), and β -III tubulin (1:500) was at room temperature for 30 min after permeabilization. After incubation with fluorophore-conjugated secondary antibodies (1:50) in PBS at room temperature for 30 min, all nuclei were counterstained with 4,6-diamidino-2-phenylindole (0.5 µg/ml) for 10 min, and then the coverslips were mounted on slide glasses with VectorShield (Vector laboratory, Burlingame, CA).

Immunoblots

Protein lysates were prepared in the lysis buffer as described previously (Horiuchi et al., 2006). Twenty µg of protein from each sample was size-fractionated by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane (Schleicher & Schnell, Keene, NH) and probed with primary antibodies for phospho-CREB (1:1000), phospho-AKT (1:1000), or phospho-ERK1/2 (1:1000) for 1 h. Full range recombinant Rainbow Molecular Weight Markers (Amersham Biosciences, Piscataway, NJ) were used as a reference for molecular sizes. Immunoreactive signals were detected by enhanced chemiluminescence according to the manufacture's protocol (Amersham Biosciences). Total CREB, AKT and ERK1/2 were detected with anti-CREB (1:1000), anti-AKT (1:1000), and anti-ERK1/2 antibody (1:1000) in the same membrane used for anti-phospho-CREB, phospho-AKT, and phospho-ERK1/2 antibodies, respectively, after deprobing. Equal protein loading was confirmed by subsequent probing with the mouse monoclonal antibody against GAPDH in each experiment. Immunoreactive signals were quantified by densitometry of the films using the image analysis software, ImageJ (<http://rsweb.nih.gov/ij/>).

Caspase activity assay

Cells were homogenized in the lysis buffer (100 mM HEPES; 10% (w/v) sucrose; 0.1% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; 10 mM dithiothreitol; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin; 1 µg/ml pepstatin; 5 µg/ml leupeptin) (Moran et al., 1999). The protein lysates were stored at -80°C until use as a 1:1 (v/v) mixture with glycerol. Caspase activity was measured by the fluorometric method; protein samples (10 µg) were incubated with the fluorogenic substrate, acetyl-Asp-Glu-Val-Asp- α -(4-methylcoumaryl-7-amide) (12.5 µM) (Ac-DEVD-AMC, Peptides international, Louisville, KY) in 250 µl of the lysis buffer, and cleavage of Ac-DEVD-AMC was monitored by a multiplate spectrofluorometer, Gemini EM (Molecular devices, Sunnyvale, CA) for 60 min at 25°C . The DEVD-cleavage activity was expressed as delta RFU (relative fluorescence unit)/µg protein/h

BrdU-incorporation assay

OPCs cultured in 60 mm dishes were exposed to a 4 h BrdU pulse (10 µM) just prior to harvesting. The trypsinized cells were collected in GM and resuspended into 1.5 ml phosphate-buffered saline (PBS). After fixation by 70% (v/v) ethanol at -20°C for

overnight, 5×10^4 cells were washed with 1 ml of the washing buffer (0.1% (w/v) BSA in PBS), and denatured by 2N HCl at room temperature for 20 min. After washing once with this buffer, the cells were incubated in 0.1 M sodium borate at room temperature for 2 min to neutralize any residual acid. Cells that had incorporated BrdU following incubation were identified with a FITC-conjugated mouse anti-BrdU monoclonal antibody at room temperature for 20 min in the dilution buffer (0.1% (w/v) BSA, 0.5% (v/v) Tween-20 in PBS) followed by wash with the washing buffer. The labeled cells were detected in the green (FL1) channel of a flow cytometer, CyAn-ADP (Dako cytometry, Carpinteria, CA). FITC-conjugated mouse monoclonal IgG1 was used as isotype control.

Time-lapse imaging

Mouse and rat OPCs were seeded on poly-D-lysine-coated 24-well plates at 5×10^4 cells/well. Time-lapse phase contrast images of the selected fields were taken at 5 min intervals for 12 hr through a Plan Fluor 10x objective lens by the Nikon Perfect focus system (Nikon, Tokyo, Japan) equipped with an environmental chamber. Ten cells were randomly picked in the serial images, and averaged migration velocity of each cell during each 5 min was calculated from the changes in the coordinates of the cell soma using Velocity (Improvision, Lexington, MA), an image analysis software.

MTT assay

Cell viability was estimated by the enzymatic conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan crystals in live cells. Produced formazan was dissolved in dimethyl sulfoxide at 90 min after addition of MTT (0.5 mg/ml) to the culture medium, and quantified by a spectrophotometer or a microplate reader at 560 nm.

Live/Dead cell assay

Cells in 96-well plates were treated with or without forskolin (FSK, 20 μ M) for 48 h. Then, 10 times concentrated staining solution containing 6-carboxyfluorescein diacetate (6-CFDA, 50 μ M) and propidium iodide (PI, 10 μ g/ml) was added to each well. After incubation for 5 min at 37°C, digitized fluorescence images of the cells were captured with an inverted fluorescence microscope (IX70, Olympus, Tokyo, Japan). Live cells positive for 6-CFDA and dead cells whose nuclei were positive for PI were counted by the off-line analysis of the images.

Statistical analysis

Data are presented as mean $\pm$ SD unless otherwise noted. Statistical significance was determined by two-tail ANOVA followed by Student-Newman-Keuls post hoc test.

Results

Mouse NG2-positive cells require forskolin to survive and differentiate into oligodendrocytes

Mixed glial cells from the brains of single PN0-PN2 mouse neonates were expanded in GM for 5-7 days until they reached subconfluence in 10 cm culture dishes (approximately 1.5×10^6 cells per dish). After trypsinization, resulting cell suspensions were labeled with anti-NG2 antibody followed by FITC-conjugated anti-rabbit IgG antibody and nuclear staining for dead cells with PI. Although the anti-NG2 rabbit polyclonal antibody used in this study was raised against rat NG2, other studies have shown that the antibodies for rat NG2 successfully labeled mouse NG2⁺ cells *in vivo* and *in vitro* (Diers-Fenger et al., 2001; Ligon et al., 2006). Live NG2⁺ cells (NG2⁺/PI⁻) were defined by flow cytometry as those

distributed in the gated area of the FITC-PI bivariate scattergram (R1 in Fig. 1B), and consisting of $33 \pm 16\%$ ($n=9$) of total mixed glial cells. Using the same gating, $0.46 \pm 0.12 \times 10^6$ NG2⁺ cells ($n=9$) were isolated from the mixed glial cell suspension by MoFlo cell sorter. These mouse NG2⁺ cells at 1 day after sorting were more than 95% pure and showed a relatively homogeneous morphology; a round cell soma with 2 to 4 short processes (Fig. 1D). However, they progressively died in GM supplemented with FGF2 (5 ng/ml) and PDGF_{AA} (1 ng/ml), which is sufficient for survival and preferential proliferation of rat OPCs (Horiuchi et al., 2006). In an attempt to determine dependency of mouse NG2⁺ cells on additional exogenous molecules, the sorted cells were plated on poly-D-lysine-coated 24-well culture plates at approximately 2.5×10^4 cells/well, and cultured in GM supplemented with FGF2 (5 ng/ml), PDGF_{AA} (1 ng/ml), and varying concentrations of FSK (0, 1, 10, and 100 μ M). At 4 days in culture, mouse NG2⁺ cells had survived and proliferated only in cultures containing FSK at concentrations of 10 μ M or higher (Figs. 1E-H). Purified mouse NG2⁺ cells were thereafter routinely expanded in GM supplemented with 20 μ M FSK for 10-12 days to obtain $5.6 \pm 0.12 \times 10^6$ cells ($n=9$). Immunocytochemical analysis confirmed that these NG2⁺ cells expanded in the presence of FSK consisted of more than 90% NG2⁺ cells, less than 2% GFAP⁺ cells, and neither β -III tubulin⁺ nor CD11b⁺ cells. However, only 25% of mouse NG2⁺ cells weakly expressed A2B5 antigen, a typical surface marker for rat OPCs (Raff et al., 1983) (Figs. 2A and 2E).

For comparison with mouse NG2⁺ cells, we isolated rat NG2⁺ cells from PN0-PN2 rat brains by the same flow-based method as used for mouse NG2⁺ cells, and found that purified rat NG2⁺ cells were 99% positive for A2B5. On the other hand, 83% of immunopanning-purified A2B5-positive rat OPCs co-expressed NG2 (Figs. 2C and 2E). Thus, unlike mouse NG2⁺ cells, rat NG2⁺ cells almost overlapped well-characterized A2B5⁺ OPCs (i.e. rat NG2⁺/A2B5⁺ OPCs) which proliferated preferentially in GM without addition of FSK as reported elsewhere (Itoh et al., 2002, Horiuchi et al., 2006).

In addition to the difference in A2B5 antigen expression, 28% of mouse NG2⁺ cells were O4-positive in GM plus FSK, while only 5% of rat NG2⁺ OPCs were O4-positive in the absence of FSK (Figs. 2B, 2D, and 2E). FSK is known to promote differentiation of oligodendrocytes (Raible and McMorris, 1989). However, addition of 20 μ M FSK to rat A2B5⁺ cultures increased O4-positive population by at most 6%. Moreover, neither galactocerebroside- nor myelin basic protein (MBP)-positive cells were detected in both mouse and rat NG2⁺ cell cultures maintained in GM plus FSK (Fig. 2E).

In order to determine the ability of mouse NG2⁺ cells for *in vitro* differentiation into mature oligodendrocytes, mouse NG2⁺ cells and rat NG2⁺ cells were transferred to DM. At 5 day in DM, 90 % of mouse NG2⁺ cells had become O4-positive and 65% expressed MBP, while 95% and 85% of rat NG2⁺ cells were positive for O4 and MBP, respectively (Fig. 3). Thus, we concluded that, as far as could be determined by the ability of mouse NG2⁺ cells for *in vitro* differentiation into MBP-positive oligodendrocytes, purified mouse NG2⁺ cells proliferating in GM plus FSK represented a cellular population comparable to rat NG2⁺ OPCs, and therefore can be considered mouse OPCs (mouse OPCs thereafter).

Purified mouse NG2⁺ OPCs were less motile than rat OPCs *in vitro*

Isolated mouse NG2⁺ OPCs had shorter processes than rat NG2⁺ OPCs in the same culture condition (Compare Figs. 2A and 2C). This morphological difference might be associated with their migration ability. As shown in Figure 4, a quantitative analysis of migration ability of rat and mouse OPCs confirmed that the average velocities and the maximum velocities of mouse OPCs during 12 h were significantly lower than those of rat OPCs. The lesser motility of mouse OPCs was unlikely to be an effect of added FSK, because addition of FSK to rat OPC cultures did not inhibit their motility (Fig. 4D).

Forskolin partially inhibited apoptosis of mouse OPCs, which was mediated by the intrinsic apoptotic pathway

In the absence of FSK, mouse OPCs died by apoptosis, as indicated by remarkable induction of caspase-3-like protease activity. FSK partially but significantly inhibited cell death as well as caspase-3-like protease activity in wild-type mouse OPCs (Fig. 5). In contrast, rat OPCs demonstrated a much lower incidence of cell death, and lower caspase-3-like protease activity, even in the absence of FSK (Fig. 5). The intrinsic apoptotic pathway is substantially inactivated in *bax*- and *bak*-double deficient (*bax/bak* DKO) cells (Wei et al, 2001). Even in the absence of FSK, the NG2⁺ OPCs obtained from *bax/bak* DKO mice survived and proliferated without apoptosis, and showed negligible caspase-3-like protease activity, indicating the involvement of the intrinsic apoptotic pathway in apoptosis of mouse OPCs in the absence of FSK (Fig. 5).

On the other hand, FSK similarly inhibited proliferation of both mouse and rat OPCs. BrdU-incorporation assay revealed that $52 \pm 3\%$ of mouse OPCs, and $49 \pm 7\%$ of rat OPCs were labeled after a 4 h BrdU pulse at 24 h after incubation in GM alone, and that there was no significant difference in the BrdU-positive percentages between mouse and rat OPCs. In the presence of FSK, BrdU-incorporated cells were significantly decreased to $36 \pm 7\%$ ($p < 0.01$) and $36 \pm 10\%$ ($p < 0.01$) in mouse and rat OPC cultures, respectively (Fig. 5I). These results indicated that, even in the absence of FSK, mouse OPCs proliferated at a similar rate to that of rat OPCs, but more mouse OPCs died than those generated due to activation of the intrinsic apoptotic pathway after isolation from other glial populations. Thus, a survival promoting effect of FSK was attributed to inhibition of the intrinsic apoptotic pathway, but not to effects on cell proliferation.

cAMP but not Epacs promotes survival of mouse OPCs

The pro-survival effect of FSK on mouse OPCs was mediated by up-regulation of cAMP signaling. As shown in Fig. 6, a cell permeable analogue of cAMP, N⁶, 2'-O-dibutyryl cAMP (db-cAMP), significantly promoted survival of mouse OPCs, although db-cAMP was not as effective as FSK at the same concentration. cAMP activates not only cAMP-dependent protein kinase (PKA), but also cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs or Epacs) (de Rooij et al., 1998). In contrast to db-cAMP, 8-(4-chlorophenylthio)-2'-O-methyladenosine-3', 5'-cyclic monophosphate (8-CPT-2Me-O-cAMP), a cell-permeable cAMP analog selective for Epac activation, did not have a significant effect on survival of mouse NG2⁺ OPCs at 0.1 mM (Fig. 6). However, 8-CPT-2Me-O-cAMP at higher concentrations than 0.1 mM activated the PKA pathway as well in mouse OPCs, and promoted their survival. These results ruled out a major contribution of Epacs to the cAMP-mediated pro-survival effect.

Basal phosphorylation levels of cAMP-response element binding protein (CREB) and ERK were lower in mouse OPCs compared with rat OPCs

We explored the molecular basis of the differing survival dependency of mouse OPCs vs rat OPCs, particularly focusing on the CREB, MEK/ERK, and AKT pathways, all of which are known to be involved in cell survival. Most notably, basal phosphorylation levels of CREB and ERK in mouse OPCs were significantly lower than those in rat OPCs (Figs. 7A, B). However, when examined at 24 h after treatment, both FSK and db-cAMP had no remarkable effects on the basal phosphorylation levels of CREB and ERK2 in mouse and rat OPCs (Figs. 7A, B). In contrast to the prolonged effects, FSK transiently up-regulated phosphorylated CREB in both mouse and rat OPCs with a peak at around 1 h (Data not shown). This transient activation was mediated by cAMP, because db-cAMP also transiently up-regulated phosphorylation levels of CREB (Figs. 7C, D). On the other hand, we did not observe transient activation of ERK2 and AKT after addition of either FSK or db-cAMP

(Figs. 7C, D). Collectively, these results suggest the involvement of cAMP-mediated CREB signaling in the survival promoting effect of FSK on mouse OPCs.

Discussion

In this study, we have established a method to purify and expand mouse OPCs from the brain of a single neonatal mouse *in vitro*, and compared their characteristics to those of rat NG2⁺ OPCs. NG2 CSPG is recognized as a surface marker of OPCs in various species (Nishiyama et al., 2009; Wilson et al., 2006), which is supported by intensive *in vivo* and *in vitro* studies (Stallcup and Beasley, 1987, Nishiyama et al., 1996, Diers-Fenger et al., 2001). In agreement with these prior studies, we first confirmed that mouse NG2⁺ cells isolated from neonatal brains are comparable to rat NG2⁺ OPCs with respect to their ability for differentiation into myelin-producing oligodendrocytes in our culture condition. However, there were notable differences in survival dependency, morphology, and motility between mouse and rat NG2⁺ OPCs under the same culture condition. This difference could be attributable to different developmental stages of the oligodendroglial lineage between mouse and rat NG2⁺ OPCs. Indeed, we observed more O4-positive population in the mouse OPC cultures than in the rat OPC cultures, suggesting that, in our *in vitro* condition, mouse NG2⁺ OPCs are maintained at a relatively more advanced developmental stage than rat NG2⁺ OPCs.

No neuronal differentiation was observed in our purified NG2⁺ cell cultures from mice and rats *in vitro*. It is still controversial, however, whether the NG2⁺ population in the CNS parenchyma consists of a homogeneous population committed only to the glial lineage *in vivo* (Reviewed by Nishiyama et al., 2009). Nishiyama and her colleagues have demonstrated that NG2⁺ cells generate not only oligodendrocytes but also astrocytes in the gray matter using NG2creBAC transgenic mice (Zhu et al., 2008). Moreover, Trapp's group demonstrated that pre-OPCs surface positive for NG2 but negative for A2B5 produce NG2⁺/A2B5⁺ OPCs in the developing rat brain, but suggested that these pre-OPCs may not be yet committed to the oligodendroglial lineage (Baracska et al., 2007). In support of the neuronal fate of NG2⁺ population, Gallo's group demonstrated that NG2⁺ cells in the postnatal subventricular zone gave rise to GABAergic interneurons, olfactory interneurons and oligodendrocytes in response to region-specific cue(s) (Aguirre et al., 2004a and b). A fate mapping study from our group have also demonstrated neurogenesis as well as astrogenesis from a subpopulation of early postnatal NG2⁺ cells whose transgene promoter of myelin proteolipid protein is active (Guo et al., 2009). We speculate that B104CM used in this study contains molecular cue(s) which preferentially drive NG2⁺ cells to the oligodendroglial lineage. In support of this, B104CM has been established to direct multipotent neural progenitor cells of various species including rats, mice, canines, and swine to the oligodendroglial lineage (Zhang et al., 1998a and b; Vitry et al., 1999; Smith and Blakemore, 2000). More significantly, mouse "neurospheres" consisting of multipotent neural progenitor cells can be differentiated into "oligospheres", the cell aggregates rich in cells of the oligodendroglial lineage, by treatment with B104CM (Vitry et al., 1999; Chen et al., 2007). It remains undetermined, however, which factors in B104CM are responsible for the preferential induction and/or selective expansion of OPCs by B104CM.

The combination of B104CM and FGF2 provides rat OPCs with sufficient *in vitro* conditions for their survival and selective proliferation without lineage progression (Itoh et al., 2002, Feldman et al., 2008), but the same medium fails to maintain purified mouse OPCs. The results of BrdU-incorporation indicated that mouse OPCs were still cycling in B104CM plus FGF2 alone, but that a large proportion of them could not survive until next division. Given that PDGF_{AA} is present in B104CM (Asakura et al., 1997; our unpublished observation), a simple explanation could be that some factor(s) other than PDGF_{AA} and

FGF2 are also essential for survival of mouse OPCs in B104CM. Since B104 is derived from rat neuroblastoma (Bottenstein et al., 1988), the bioactivity of this unidentified factor(s) could be species-specific (i.e. acting on rat OPCs but not on mouse OPCs). On the other hand, Ray and Gage (2006) also pointed out the difference between mice and rats in the response of adult neural stem/progenitor cells to exogenous mitogens and heparin. Exogenous heparin inhibits the mitogenic activity of FGF2 on rat neural stem/progenitor monolayer cultures but potentiates the action of FGF2 on mouse neural stem/progenitor monolayer cultures. Thus, it is conceivable that mouse OPCs express different sets and/or amounts of heparin and heparan sulfate proteoglycans than rat OPCs, resulting in differences in FGF2 signaling between OPCs of these two rodent species.

We previously demonstrated that the MEK-ERK pathway is strongly activated in rat OPCs maintained in B104CM plus FGF2 (Horiuchi et al., 2006). The commercial antibody against phospho-ERKs used both in the present study, and in our prior study, was derived from rabbits immunized with a synthetic phospho-peptide of human p44 ERK containing the phosphorylation site (Thr202/Tyr204). Since the at least 60 amino acid stretch flanking this immunogenic region is completely identical in human, mouse and rat ERK1/2, it is quite unlikely that lower immunoreactive signals for phospho-ERKs in mouse OPCs than those in rat OPCs was due to the difference in antigenicity of the phosphorylation sites between the two rodent species. We therefore concluded that ERKs were less activated in mouse OPCs in the same culture medium compared with rat OPCs. Although cAMP is known to down-regulate phospho-ERK by inhibiting Raf1, one of the MAP kinase kinases at the apical level of the MEK-ERK pathway, in certain cell types (Schmitt and Stork 2002), reduction in phosphorylated ERK2 levels after addition of FSK in both mouse and rat OPCs was mild compared to the clear difference in basal levels of phosphorylated ERK2 between mouse and rat OPCs. These results further support the idea that trophic support of B104CM plus FGF2 is insufficient for mouse OPCs, thus resulting in less activation of ERK compared with rat OPCs.

Lack of sufficient trophic support normally triggers activation of the intrinsic apoptotic pathway (Putchá et al., 1999; Hieronymus et al., 2000). Indeed, the incidences of cell death and caspase-3-like protease activity were significantly higher in mouse OPCs than in rat OPCs. As observed in the trophic factor deprivation models of other cell types (Wei et al., 2001), OPCs lacking functional BAX and BAK confirmed that the intrinsic apoptotic pathway was solely responsible for apoptosis of mouse OPCs maintained in B104CM plus FGF2. FSK partially inhibited this activation of the intrinsic pathway. Several studies have demonstrated cross-talk between cAMP signaling and pro-survival signaling pathways such as the MEK-ERK and the phosphatidylinositol 3-kinase-AKT pathways (Hecquet et al., 2002; Mei et al., 2002; Schmitt and Stork, 2002). However, we failed to detect additional activation of ERK1/2 and AKT by db-cAMP and FSK in mouse OPCs. It is therefore unlikely that cross-talk between these pathways contributes to the pro-survival effect of enhanced cAMP signaling. Although direct inhibition of mitochondrion-mediated apoptosis by cAMP signaling has been reported in neurons after nerve growth factor deprivation (Putchá et al., 1999), the molecular link between cAMP signaling and inhibition of the intrinsic apoptotic pathway in mouse OPCs remains to be clarified.

cAMP signaling has been reported to have at least two distinct effects on the oligodendroglial lineage; inhibition of OPC proliferation and accelerated differentiation of oligodendrocytes (Raible and McMorris, 1993). In mouse OPCs maintained in B104CM plus FGF2, however, addition of FSK inhibited proliferation, but did not induce myelin components. Even in rat OPC cultures, as far as they were maintained in B104CM plus FGF2, FSK increased O4 antigen-positive populations from 5% to only 6% of the total OPCs (Fig. 2E) despite its inhibition of the cell cycle. Our results suggest that the effects of

cAMP signaling on survival, progression of the cell cycle, and the endogenous schedule of differentiation depend on the strength of signal inputs from mitogens present in the employed *in vitro* condition.

We noticed two distinct activity modes of CREB phosphorylation in OPCs; basal activity and transient up-regulation. Most prior studies of OPC CREB signaling have focused on the transient up-regulation by elevation of cAMP, cytosolic calcium concentration (Pende et al., 1997), or activation of receptor tyrosine kinase-mediated signaling (Johnson et al., 2000). In fact, addition of FSK or db-cAMP induced a transient up-regulation of phosphorylated CREB, which was similarly observed in both mouse and rat OPCs. However, our data point toward the importance of the basal phosphorylation level of CREB on OPC survival rather than transient up-regulation of phospho-CREB levels immediately following such perturbations. Basal CREB phosphorylation was higher in rat OPCs than in mouse OPCs, suggesting that a threshold level of basal CREB activity may be required to promote survival of OPCs. The significance of basal CREB activity in OPC survival, and responsible upstream CREB kinases such as protein kinase A (Shiga et al., 2005a), protein kinase C (Shiga et al., 2005b), and ribosomal S6 kinases (Xing et al., 1996), need to be determined in future experiments.

In summary, our results demonstrate that there is a qualitative difference between mouse NG2⁺ OPCs and rat NG2⁺ (NG2⁺/A2B5⁺) OPCs particularly in response to B104CM plus FGF2, an optimum *in vitro* condition for rat OPCs. Mouse NG2⁺ OPCs require additional activation of cAMP signaling for survival to inhibit activation of the intrinsic apoptotic pathway in B104CM plus FGF2.

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Figure 1.

A, B, Representative flow cytometric results of mouse mixed glial cells unlabeled (**A**) and labeled with anti-NG2 antibody (FITC) and PI (**B**). The cell population distributed within gate R1 was isolated by MoFlo cell sorter to obtain pure NG2⁺/PI⁻ cells. **C, D,** Representative phase contrast images of mixed glial culture before sorting (**C**) and of NG2⁺/PI⁻ cells at 24 h after cell sorting (**D**). **E-H,** phase contrast images of purified NG2⁺ cells at 1 h after plating (**E**), and those cultured for 4 days in the presence of 1 (**F**), 10 (**G**), and 100 μM (**H**) of forskolin (FSK). Addition of 10 or 100 μM of FSK to the culture medium enabled NG2⁺ cells to survive and proliferate, while almost all of NG2⁺ cells died within 4 days in medium containing FSK at 1 μM (**F**) or less (not shown). Scale bars; 100 μm in **C** and **D**, and 50 μm in **E-H**.

Figure 2.

A-D, Immunocytochemistry of the primary cultures of mouse (**A**, **B**) and rat NG2-sorted cells (**C**, **D**). Cells maintained in GM were stained with anti-NG2 antibody (green in **A-D**), A2B5 (red in **A**, **C**), and O4 (red in **B**, **D**). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (blue). Scale bar; 50 μ m. **E**, Percentages of the cell populations immunopositive for NG2, A2B5, O4, GalC, GFAP, CD11b, or β -III tubulin (β IIIT) in rat A2B5-immunopanned, rat NG2-sorted, and mouse NG2-sorted cell cultures. Rat cells were cultured in either GM or GM supplemented with 20 μ M FSK (GM+FSK), while mouse NG2⁺ cells were maintained only in GM+FSK, because they could not survive without FSK.

Figure 3.

A-D, Mouse (**A**, **B**) and rat NG2-sorted (**C**, **D**) primary cultures were stained with anti-GalC (green in **A**, **C**), O4 (red in **A**, **C**), anti-MBP (green in **B**, **D**), and anti-NG2 (red in **B**, **D**) antibodies at 5 day after *in vitro* differentiation. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (blue). Scale bar; 50 μ m. **E**, Percentages of the cell populations immunopositive for NG2, O4, GalC, MBP, or GFAP in mouse and rat cultures at 5 day after *in vitro* differentiation. Mouse cells were expanded in GM plus 20 μ M FSK (GM+FSK), and rat cells were expanded in either GM or GM+FSK before switching culture medium to DM.

Figure 4.

Time-lapse phase contrast images of mouse (A) and rat OPCs (B). Phase contrast images of the exact same fields were taken at 5 min intervals with the time-lapse observation system from 5 min to 4 h after adding fresh medium. Representative image fields at 10 min intervals during first 55 min are shown. Individual cells could be traced easily during the experiments (arrows). Mouse OPCs bearing short processes were less motile than rat NG2⁺ OPCs which actively migrated along their long bipolar or tripolar processes. Scale bar; 50 μm. C, Averaged velocities of randomly selected 10 rat OPCs in GM or GM supplemented with FSK (20 μM; GM+FSK) and 10 mouse OPCs in GM+FSK were calculated every 5 min for 4 h. D, A bar graph shows average velocities (Average, open bars) and the maximum velocities (Maximum, closed bars) of mouse OPCs in GM+FSK and rat OPCs in GM or GM +FSK during 12 h. ** indicates p < 0.01 when compared with mouse OPCs.

Figure 5.

A-F, Representative fluorescence images of OPC cultures stained with 6-CFDA (green) and PI (red). Wild-type mouse OPCs (**A**, **B**), *bax*- and *bak*-double deficient mouse OPCs (**C**, **D**), and rat OPCs (**E**, **F**) were incubated in GM alone (**A**, **C**, **E**) or GM plus FSK (20 μM) (**B**, **D**, **F**) for 48 h, and subjected to the Live/Dead cell assay. **G**, Summarized result of the Live/Dead assay. Bar graphs show percentages of PI⁺ dead cells to total cells in the cultures of wild-type mouse OPC (WT), *bax*- and *bak*-double deficient mouse OPC (*bax/bak* DKO), and rat OPC (rat) after a 48 h treatment with GM alone (control) or GM plus FSK (20 μM, FSK20). **H**, Caspase-3-like protease activity in OPCs from wild-type mice (WT), *bax*- and *bak*-double deficient mice (*bax/bak* DKO) and rats in the presence or absence of FSK. DEVD cleavage activity was almost undetectable in *bax*- and *bak*-double deficient OPCs. Twenty four h treatment with FSK (20 μM, FSK20) significantly decreased DEVD cleavage activity in WT OPCs. * and ** indicate $p < 0.05$ and $p < 0.01$ in comparison with the control cultures of wild-type mouse OPCs, respectively (**G** and **H**). **I**, BrdU-positive cells in rat and mouse OPC cultures immediately after a 4 h BrdU single pulse were counted at 24 h after incubation in fresh medium. Data are presented as percentages of total cell numbers. ** indicates $p < 0.01$.

Figure 6.

The membrane-permeable agonist of cAMP, N⁶, 2'-*O*-dibutyryl-cAMP (db-cAMP), but not the Epac-selective agonist of cAMP, 8-CPT-2Me-*O*-cAMP, had pro-survival effects on mouse NG2⁺ OPCs. Cells were cultured in the presence of FSK (0.1 mM), N⁶, 2'-*O*-dibutyryl-cAMP (db-cAMP, 0.1 mM), or 8-CPT-2Me-*O*-cAMP (8CPT-cAMP, 0.1 mM), for 48 h, and their viabilities were measured by MTT assay. Data are presented as percents of O. D. 560 nm of the cells maintained in the medium alone. ** indicates $p < 0.01$ in comparison with the control cultures.

Figure 7.

A, Basal phosphorylation levels of CREB and ERK2 were lower in mouse OPCs than in rat OPCs in GM alone (C, control). However, neither FSK nor db-cAMP increased basal phosphorylation levels of CREB, ERK2 or AKT in both mouse and rat OPCs at 24 h after treatments. Protein samples from mouse and rat OPCs maintained in GM (C) or in GM containing FSK (20 μ M) (F) or db-cAMP (0.3 mM) (db) for 24 h were immunoblotted for phosphorylated forms of CREB, ERK1/2, and AKT, and for total-CREB, ERK1/2, and AKT. **B**, Densitometric analysis of the triplicated immunoblots shown in **A**. Levels of phosphorylated CREB, ERK2, and AKT were calculated as ratios to the corresponding total protein levels, and levels of total proteins were calculated as ratios to GAPDH protein levels. Then the calculated values are presented as percentages of the values at mouse control. **C**, Phosphorylation levels of CREB, ERK2, and AKT in mouse OPCs at 1 h after treatments with FSK and db-cAMP. Protein samples from mouse OPC cultures before (0 h) and at 1 h after treatment with GM in the presence of FSK (F, 20 μ M) or db-cAMP (db, 0.3 mM) were subjected to immunoblotting for phospho-CREB, total-CREB, phospho-ERK1/2, total-ERK1/2, phospho-AKT, and total-AKT. **D**, Summarized densitometric results from more than three independent blots. Levels of phosphorylated CREB, ERK2, and AKT at 1 h after treatments were calculated as ratios to the signals for corresponding total proteins. Levels of total proteins were calculated as ratios to GAPDH protein levels. Then the calculated values are presented as percentages of the averaged values of control cultures at 1 h. ** and * indicate $p < 0.01$ and $p < 0.05$, respectively.