Differential Regulation of Basic Helix-Loop-Helix Factors Mash1 and Olig2 by β-Amyloid Accelerates Both Differentiation and Death of Cultured Neural Stem/Progenitor Cells*

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Despite increased neurogenic differentiation markers in the hippocampal CA1 in Alzheimer disease, neurons are not replaced in CA1 and the neocortex in the disease. β-Amyloid (Aβ) might cause deterioration of the brain microenvironment supporting neurogenesis and the survival of immature neurons. To test this possibility, we examined whether Aβ alters the expression of cell fate determinants in cerebral cortical cultures and in an Alzheimer disease mouse model (PrP-APPsw). Up-regulation of Mash1 and down-regulation of Olig2 were found in cerebral cortical cultures treated with Aβ (1–42). Mash1 was expressed in nestin-positive immature cells. The majority of Mash1-positive cells in untreated cortical culture co-expressed Olig2. Aβ increased the proportion of Olig2-negative/Mash1-positive cells. A decrease in Olig2+ cells was also observed in the cerebral cortex of adult PrP-APPsw mice. Cotransfection experiments with Mash1 cDNA and Olig2 siRNA revealed that overexpression of Mash1 in neurosphere cells retaining Olig2 expression enhanced neural differentiation but accentuated death of Olig2-depleted cells. Growth factor deprivation, which down-regulated Olig2, accelerated death of Mash1-overexpressing neurosphere cells. We conclude that cooperation between Mash1 and Olig2 is necessary for neural stem/progenitor cells to develop into fully mature neurons and that down-regulation of Olig2 by Aβ in Mash1-overexpressing cells switches the cell fate to death. Maintaining Olig2 expression in differentiating cells could have therapeutic potential.

Adult neural stem/progenitor cells continue to generate neurons in the brain throughout life, and therefore, there are high expectations that it may be possible to repair the nervous system in neurodegenerative diseases such as Alzheimer disease by stimulating endogenous progenitor cells or by stem cell transplantation. Neural stem/progenitor cells have successfully generated new neurons in vitro (1); however, neurogenesis in vivo appears to be limited to the subventricular zone and the subgranular zone of the adult intact brain (2). The environment of other regions of the adult brain, including the neocortex, may be restrictive for the differentiation of progenitors or the survival of newly formed neurons. If the environmental restriction could be reduced, new neurons could be generated from progenitors and could survive in the adult brain. In fact, brain injuries induce proliferation of progenitors in the subventricular zone and the subgranular zone and sometimes cause migration of newly generated neurons to injured sites of the adult brain (2). A specific type of injury also appears to induce in situ neurogenesis in the adult neocortex that does not normally undergo neurogenesis (3). In Alzheimer disease (AD),2 immature neuronal marker proteins are increased in the subgranular zone and the CA1 region of the hippocampus (4). However, the increased neurogenesis in the AD brain is not sufficient to repair the nervous system because progressive neuronal loss occurs in CA1 of AD patients. Alterations of the microenvironment in the AD hippocampus may affect the fate of immature neurons. Because β-amyloid (Aβ), of which deposits are one of the hallmarks of AD, plays an important role in the early pathogenesis of AD (5), it is reasonable to speculate that Aβ alters the brain microenvironment to make it toxic to newborn neurons. AD mouse models and stem cell culture may be useful tools for examining this possibility. However, contradictory findings have been reported regarding whether hippocampal neurogenesis in AD mouse models is impaired or enhanced; most AD mouse models (PrP-APPsw Tg, PDGF-APPV717F Tg, PS1P117L Tg, PS1M146V knockin) show impaired hippocampal neurogenesis (6–11), but in one AD mouse model (PDGF-APPsw Tg), increased neurogenesis was reported (12). The in vitro effects of Aβ peptide on the proliferation and differentiation of neural stem/progenitor cells are also controversial (9, 13–15). Thus, the question of whether the brain microenvironment is altered by Aβ, rendering it toxic to progenitor cells and immature neurons, still remains unanswered.

Neocortical neurogenesis has not been reported at all in AD patients. However, in an AD mouse model (PDGF-
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β-Amyloid that has impaired hippocampal neurogenesis, bromodeoxyuridine (BrUrd)-incorporating mature neurons (BrUrd +/NeuN+ cells) have been found in non-neurogenic regions throughout the adult central nervous system, including the cerebral cortex (6). Therefore, the possibility of neocortical neurogenesis in AD cannot be ruled out. The molecular mechanisms underlying disease-induced cell proliferation and differentiation of progenitors in the neocortex are largely unknown.

To address these issues, we examined the expression of basic helix-loop-helix factors, which are involved in neurogenesis during development, in Aβ-treated cultures, and in the cerebral cortex of an AD mouse model (PrP-APPsw, Tg2576). We also examined whether the altered expression of these genes leads to the stimulation of neuronal differentiation or death. The results presented here demonstrate the up-regulation of Mash1 and down-regulation of Olig2 in cerebral cortical cultures treated with Aβ. Mash1 and Olig2 are basic helix-loop-helix transcription factors involved in initiating neurogenesis and oligoden-}

Experimental Procedures

Cell Culture—For neuronal cell culture, cerebral cortices dissected from day E14 embryonic rats were dissociated by incubation with 0.08% trypsin, 0.008% DNase I at 37 °C for 10 min and passed through a 62-µm nylon mesh. The cells (10^6 cells/dish for the preparation of RNA or protein) were seeded in gelatin-polyornithine-coated dishes of 3.5- or 6-cm diameter, respectively, and were cultured for 3 or 6 days in minimum essential medium with N2 supplement, and neutralized. The cells were seeded onto gelatin-polyornithine-coated dishes at a density of 10^4 cells/cm^2 and fed for 16 h in the above medium for further transfection experiments.

Treatment with Aβ Peptides—Aβ(1–42) and synthetic peptides with the reverse sequence of Aβ(1–42) or Aβ(1–40) (Bachem Inc.) were dissolved at 250 µM in 0.05 N HCl, filtered through a 0.45-µm membrane filter, diluted with minimum essential medium with N2 supplement, and neutralized. The peptide solution (5 µM) was added to the 3 or 6 DIV cultures immediately after preparation.

Mice—Three pairs of 15–21-month-old heterozygous Tg2576 (PrP-APPsw) mice and wild-type littermates were purchased from Taconic Farm, Inc. Mice were killed by anesthesia overdose and perfused transcardially with saline followed by ice-cold phosphate-buffered 4% paraformaldehyde. Brains were post-fixed in phosphate-buffered 4% paraformaldehyde for 24 h at 4 °C. After the cryoprotection with 20% sucrose, brains were sectioned coronally at 10 µm through the entire hippocampus on a freezing microtome.

cDNA Macroarray—Total RNA from cultured cortical neu-}

Northern Blot Analysis—Poly(A)+ RNA from cultured cortical neurons or neurosphere cells was isolated using a MicroFastTrack 2.0 kit (Invitrogen). Aliquots of 2 µg of poly(A)+ RNA were denatured, electrophoretically fractionated on a 1.4% agarose, formaldehyde gel, and transferred to a nylon membrane. Hybridization was performed in a solution containing cloned cDNA labeled with [32P]dCTP according to the manufacturer’s instructions (Clontech, PT3140-1). [32P]-Labeled cDNA probes were hybridized to separate Atlas Rat 1.2 Array (Clontech) membranes according to the manufacturer’s instructions (Clontech, PT3140-1). Radioactivity on the array membrane was measured with a Bioimage analyzer BAS 2500 (Fuji Film) and analyzed with ArrayGauge Version1.2 (Fuji Film). Genes in which expression levels changed >2.5-fold in signal intensity between Aβ-treated and untreated samples from each experiment were selected for further quantitative RT-PCR or Northern blotting. Each macroarray experiment was performed in duplicate.

RT-PCR Cloning of cDNA and Quantitative Real-time PCR (QRT-PCR)—First-strand cDNA was synthesized from poly(A)+ RNA of cultured cortical neurons using SuperScript II and oligo(dT) primers (Invitrogen). PCR was carried out with EOLNase enzyme mix (Invitrogen) and gene-specific primers. Each PCR product was cloned into a pCRII vector (Invitrogen). Quantitative RT-PCR analysis was performed using an iCycler iQ detection system (Bio-Rad) with EOLN}

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**TABLE 1**
List of primers for quantitative RT-PCR

| Gene      | Forward sequence                  | Reverse sequence                      |
|-----------|-----------------------------------|---------------------------------------|
| GADPH     | CTTCATCTGTCCTTTTCTCATACTG         | GCTCATTGTGCTTTTTCTCATACTG             |
| ACTB      | CTTCACCCACCTGAGAAAGGG             | CATCGGTCAAGGCTGCTGTCAGT              |
| ACTB      | CCAGGGTTGTAGTGGTTGGTAGGATGCGAAGAG | TCCAGGTTCAAGGCTGCTGTCAGT             |
| GAPDH     | CCACTCTTTCCTGCAAGCTTGCATGAC      | TCCTGCCATCTGCTTCTCACAAG              |
| NUDR      | TGAGAATGGGCGGAGTTATGTAATC         | CTGGCAGAGTTGCTTTTCTCAAAG             |
| NUDR      | CGGCGGATTGCTGCTGACTTTG           | GCTGTGAAGAAAAAGGCGAAAGTCC            |
| CPG16     | GAGGAAAAGGGAGGAGAAGAGGGAAAGG     | TCAAAAAGGTGGTCAAGAAGG                |
| Er8a2     | CGGATCTGAGGAGGAGGAGGAGGAAGTTA    | AATTCGCCGGGTCAACAAATGGGCC            |
| MYH9      | CTTACCACTCAGGAGGAGG              | CATGGACCTTGTTCTGAGGCG                |
| GAGDH     | CGGAGATGGAGGATGCTGTCGACTG         | ATTCGCCCGGTTCACAAATGGGCC             |
| ACTB      | CGGAGATGGAGGATGCTGTCGACTG         | ATTCGCCCGGTTCACAAATGGGCC             |

**Construction of Mash1**—The coding fragment of rat Mash1 cDNA was generated with RT-PCR. First-strand cDNA was synthesized using SuperScript II (Invitrogen) and gene-specific primers (nucleotides 1546–1525). PCR was carried out with EOLGase Enzyme Mix (Invitrogen) and specific primers (nucleotides 618–641 and 1546–1525). The PCR product was cloned into a pCR2.1 vector (Stratagene), and the nucleotide sequence was analyzed to confirm the authenticity of the rat Mash1 cDNA. The coding fragment of Mash1 with a c-Myc epitope was cloned into the Smal site of a pIRES-hrGFP-1a expression vector (Stratagene).

**Transfection**—Dissociated cells from 7DIV neurospheres were fed for 16 h in growth medium on gelatin-polyornithine-coated dishes. The constructs were transfected to the monolayer culture of neural stem cells with Lipofectamine 2000 according to the manufacturer’s manual (Invitrogen). To induce the differentiation of neurosphere cells, the medium was replaced with Dulbecco’s modified Eagle’s medium/F-12 containing 1% fetal bovine serum and lacking FGF2 and FGF 24 h after transfection, and neurosphere cells were cultured for an additional 24 h.

**RNA Interference**—Three types of 21-oligonucleotide siRNA for Olig2 and a scramble control (5′-CCAUUACCCUCUACGGAUGCAGTCTT-3′), designed as described on the Invitrogen or Takara webpage, respectively, were purchased from Takara. To select effective siRNA, monolayer neurosphere cells were transfected with each of 3 siRNAs and cultured for an additional 48 h. Olig2 siRNA#535 (5′-CGCAAGACUAAACUGCAUGATT-3′) was chosen as the most effective by Western blotting using anti-Olig2 antibodies.

**Immunofluorescence for Cells**—Cells were fixed with 4% paraformaldehyde for 10 min at room temperature, treated with 0.3% Triton X-100 for 10 min, blocked with 2% skim milk for 30 min, and reacted with primary antibodies for 1 h followed by a 1-h reaction with secondary antibodies. For the quantification of apoptosis, cells were incubated with 10 μM Hoechst 33342 in Dulbecco’s phosphate-buffered saline without Ca2+ and Mg2+ for 10 min. Immunofluorescence was visualized with an Olympus epifluorescence microscope. To estimate the density of Mash1-expressing cells, they were counted in the area occupied by 1000 βIII-tubulin-expressing neurons in each experiment, because they were not uniformly distributed in culture dishes but, rather, had a patchy distribution. For the characterization of Mash1-expressing cells, cells expressing lineage-specific markers were counted in at least 100 Mash1-expressing cells in each experiment. Apoptotic cells were counted in at least 300 transfected cells for each construct and in each transfection experiment.

**Immunofluorescence for Mouse Brain Sections**—Immunofluorescence of frozen mouse brain was performed according to above protocols. After immunofluorescence, brain sections were treated with 10 mM CuSO4 in 50 mM ammonium acetate buffer (pH 5.0) for 30 min for quenching autofluorescence of lipofuscin pigment inside neurons in aged mice brains (18). For the quantification of Mash1- or Olig2-expressing cells in cerebral cortices of Tg2576 and wild-type mice, they were counted on a 180,000-μm2 area within frontoparietal gray matter in each section. Quantification was performed in three sections per animal by means of Win Roof (Mitani) connected to an Olympus epifluorescence microscope.

**Western Blot Analysis**—Cells were extracted with radioimmune precipitation assay buffer containing 2 mM EDTA and protease inhibitors and centrifuged for 20 min at 14,000 rpm at 4 °C. The nuclear fractions, prepared from cultured cells using the Qproteome nuclear protein kit (Qiagen), were extracted with 2% SDS. The lysates were analyzed by SDS-PAGE (a 5–15% acrylamide linear gradient gel). After transferring to Immobilon, proteins were detected with specific antibodies using the enhanced chemiluminescence method.

**Antibodies**—The primary antibodies used were polyclonal anti-Myc-tag antibodies (MBL), a monoclonal anti-Mash1 (Pharmlingen), a monoclonal anti-nestin antibody (Rat401, Chemicon), polyclonal anti-nestin antibodies (IBL), a monoclonal anti-βIII-tubulin (TUJ1, Berkeley Antibody Co., Inc.), a monoclonal anti-GFAP (GA5, Oncogene Science), polyclonal anti-Olig2 antibodies (IBL), and a monoclonal anti-human αB-(11–28) (12B2, IBL). Secondary antibodies were Texas Red-conjugated anti-rabbit IgG (Vector), Alexa 488-conjugated goat anti-mouse IgG2a, or anti-rabbit IgG, and Alexa 594-conjugated goat anti-mouse IgG1 (Molecular Probes). Immunoadsorbed Mash1 antibody was prepared using the following protocols. The coding fragment of Mash1 with a c-Myc epitope was cloned into pGEX 4T-3 expression vector (Amerham Biosciences). Transformed BL21 cells were grown in the presence of isopropyl 1-thio-β-D-galactopyranoside, and the cell lysate was applied to GSH-Sepharose 4B column (Amerham Biosciences). After washing with phosphate-buffered saline, a monoclonal anti-Mash1 (Pharmlingen) was applied to the column, which was again washed with 5 volumes of phosphate-buffered saline. The washings were pooled and used for immunoblotting.
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TABLE 2
Aβ-induced developmentally regulated genes in cerebral cortical cells

| Gene description (accession no.) | -Fold Δ in macroarray | QRT-PCR Control | QRT-PCR Aβ |
|----------------------------------|-----------------------|-----------------|-----------|
| Ngn1 (U67777)                    | 6.03                  | (3.69 ± 1.02)e−07 | (4.12 ± 0.79)e−07 |
| Pax6 (U69644)                    | 3.14                  | (6.67 ± 0.67)e−03 | (9.01 ± 0.82)e−03 |
| Mash1 (X53725)                   | 5.32                  | (3.90 ± 0.13)e−04 | (4.01 ± 0.20)e−04 |
| NUDR (AF055884)                  | 6.44                  | (8.16 ± 0.73)e−03 | (14.99 ± 2.20)e−03 |
| Stearyl-CoA desaturase2 (U67995) | 3.32                  | (2.51 ± 0.13)e−04 | (3.52 ± 0.13)e−04 |
| GOT1 (104171)                    | 5.27                  | (1.25 ± 0.15)e+03 | (1.45 ± 0.29)e+03 |
| CPG16 (U78857)                   | 3.34                  | (6.63 ± 0.62)e−01 | (7.35 ± 1.06)e−01 |
| Erbα2 (M31177)                   | 6.59                  | (1.74 ± 0.24)e+01 | (1.56 ± 0.25)e+01 |
| Tau (X79321)                     | 3.42                  | ND               | ND        |
| MAP1B (X60370)                   | 6.16                  | ND               | ND        |
| RNB6 (U70211)                    | 9.48                  | ND               | ND        |
| MYH7 (U51463)                    | 2.96                  | (3.39 ± 0.29)e−03 | (3.07 ± 0.63)e−03 |
| TMSB4X (M34043)                  | 5.56                  | ND               | ND        |

*p < 0.05 (Student’s t test).

RESULTS

Gene Expression Results—We first identified the cell populations in our cortical cultures using antibodies against lineage-specific markers; anti-βIII-tubulin antibody for neurons, anti-nestin for immature cells, anti-GaC for oligodendrocytes, and anti-GFAP for astrocytes. Immunolabeling of lineage-specific markers revealed that our cortical cultures included a large fraction of βIII-tubulin+ neurons (90.1 ± 1.4 or 68.6 ± 0.8% of total cells in 3 or 6 DIV cultures, respectively) and a small fraction of nestin+ immature cells (13.6 ± 1.4 or 24.3 ± 4.4% of total cells in 3 or 6 DIV cultures, respectively). A few GFAP+ cells (5.8 ± 1.4% of total cells) appeared in 6 DIV cultures, but no GaC+ oligodendrocytes were detected in 6 DIV cultures. When 6 DIV cortical cultures were treated with 5 μM Aβ-(1–42), the neuronal viability began to decrease after 6 h and continuously decreased for at least up to 48 h, resulting in 91, 87, 85, and 66% of the untreated control viability after 6, 15, 24, and 48 h of treatment, respectively (19). The viability of nestin+ cells did not decrease, at least up to 15 h of treatment. RNA isolated from cortical cultures at 3 h (before neurodegeneration) and 15 h (slight neurodegeneration) after treatment with Aβ was used to generate hybridized probes for use with a nylon DNA macroarray with 1176 genes. An intensity of 30 was used as the cutoff value of the radioactive signal intensity because this was a meaningful signal higher than the background level. We used a 2.5-fold change in signal intensity between Aβ-treated and untreated samples as the cutoff line. Using these criteria, we identified 34 up-regulated genes at 3 h (early responsive) and 26 up-regulated genes at 15 h (late responsive) after Aβ treatment, respectively. Gene expression analysis of E20 embryonic rat brain using the same type of macroarray revealed that 65% of early responsive genes to Aβ and 50% of late responsive genes to Aβ were embryonic genes. To verify the array results, we performed QRT-PCT or Northern blotting. Among 13 fetal genes identified as late responsive genes to Aβ, only the Mash1 gene was thereby confirmed to be up-regulated (Table 2). The expression of other transcription factors, such as Mash2, neuroD2, and neurogenin 1, was not altered by Aβ treatment. To determine whether Aβ but not synthetic peptides with the reverse sequence of Aβ induces Mash1 expression, we compared the levels of Mash1 transcripts in cortical cultures treated with Aβ or synthetic peptides with the reverse sequence of Aβ-(1–42) (R42) or Aβ-(1–40) (R40) by QRT-PCR. Only Aβ-(1–42), but not R42 or R40, was able to induce Mash1 gene expression (the relative levels of Mash1 to β-actin transcripts were (2.37 ± 0.05)e−04, (3.17 ± 0.31)e−04, (2.19 ± 0.17)e−04, and (1.93 ± 0.19)e−04 in cortical cells treated with minimum essential medium with N2 supplement, Aβ-(1–42), R42, or R40, respectively; p < 0.05, mean ± S.E., n = 5). To examine whether there was an increase in Mash1 protein, we performed Western blotting on 3 or 6 DIV neuronal cultures. The intensity of the 34-kDa band (authentic Mash1 band) in neuronal cultures was not altered by 5 μM Aβ treatment; however, an additional immunoreactive band (about 70 kDa) appeared in the Aβ-treatment cultures disappeared when immunoadsorbed Mash1 antibody was used for detection (data not shown). To characterize the 70-kDa band, we performed Western blotting on cytosolic and nuclear fractions separated from cells treated with Aβ-(1–42) (Fig. 1A). The intensity of the 34-kDa band (authentic Mash1 band) in neuronal cultures was not altered by 5 μM Aβ treatment; however, an additional immunoreactive band (about 70 kDa) appeared in the Aβ-treated cultures but not in the control cultures. The 70-kDa band in the Aβ-treated cultures disappeared when immunoadsorbed Mash1 antibody was used for detection (data not shown). To characterize the 70-kDa band, we performed Western blotting on cytosolic and nuclear fractions separated from cells treated with Aβ-(1–42) or reverse peptide R42. The 70-kDa band appeared only in the nuclear fraction from cells treated with Aβ-(1–42) but not with R42 (Fig. 1B), indicating that the 70-kDa band may be Mash1-related. The 70-kDa band was not detected with anti-Aβ-(1–16) antibody in the Mash1 immunoprecipitate generated from Aβ-treated cells (Fig. 1C), indicating that the 70-kDa band was not a complex with Aβ. Thus, the total Mash1 protein level increased with Aβ treatment, consistent with the mRNA level. In contrast, Olig2 protein level decreased with Aβ treatment (Fig. 1A). Next, we examined whether Aβ but not reverse peptides alters the expression of Olig2 protein. Only Aβ-(1–42) but not reverse peptide R42 was able to reduce Olig2 protein in nuclear fractions from cortical cells treated with minimum essential
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FIGURE 1. Differential regulation of Mash1 and olig2 proteins by Aβ-(1–42). Rat cerebral cortical cells were seeded and cultured as indicated under "Experimental Procedures." A, three or 6 DIV cortical neurons were treated with non-aggregated Aβ-(1–42) (final concentration 5 μM) for an additional 18 h. Cell lysates were fractionated by SDS-PAGE on a 5–15% linear gradient gel and analyzed by immunoblotting with anti-Mash1, anti-Olig2, anti-GFAP, or anti-III-tubulin antibodies. The Mash1-positive band indicated by the lower asterisk corresponds to authentic Mash1 protein. The extra Mash1-positive band above 66 kDa, indicated by the upper asterisk, appeared only in lysates from Aβ-treated cells. The increase in Mash1 in the extra band and the decrease in Olig2 appeared more clearly in the Aβ-treated 3 DIV culture. B, three DIV cortical neurons were treated with Aβ-(1–42) or synthetic peptides with the reverse sequence of Aβ-(1–42) (R42) for an additional 18 h. Cytosolic and nuclear fractions from cells were separated by SDS-PAGE on a 5–15% linear gradient gel and analyzed by immunoblotting with anti-Mash1, anti-Olig2, or anti-p62 antibodies. The extra Mash1-positive band above 66 kDa (upper asterisk) and authentic band (lower asterisk) appeared in only the nuclear fraction from cells treated with Aβ-(1–42) but not with R42. C, three DIV cortical neurons were treated with Aβ-(1–42) or reverse peptide (R42) for an additional 18 h. Cell lysates were analyzed by immunoblotting with anti-Mash1 (left). Mash1 in the cell lysate was recovered by immunoprecipitation (IP) with anti-Mash1. The immunoprecipitates were then analyzed by immunoblotting with anti-Aβ-(1–16) (IBL) (right). The extra Mash1 band (arrow) was not recognized by the anti-Aβ-(1–16) in the Mash1 immunoprecipitates. D, three DIV cortical neurons were treated with various concentrations of non-aggregated Aβ-(1–42) or aggregated Aβ-(1–42) or were exposed to 50% oxygen for an additional 18 h. Cell lysates were fractionated by SDS-PAGE on a 5–15% linear gradient gel and analyzed by immunoblotting with anti-Mash1, anti-Olig2, or anti-III-tubulin antibodies. The increase in the authentic Mash1 band was clearly observed when cells were treated with 1 μM non-aggregated Aβ or with 0.5–1.0 μM aggregated Aβ. The increase of the 70-kDa immunoreactive band was only detected in cultures treated with a higher concentration (5 μM) of non-aggregated or aggregated Aβ. In contrast to Aβ, 50% oxygen exposure, which induces cell death via free radicals, did not increase the total Mash1 protein level. A decrease in Olig2 expression was observed when cells were treated with 5 μM non-aggregated or aggregated Aβ or with 50% oxygen exposure. Thus, 1 μM or a higher concentration of Aβ-(1–42), independent of the state of aggregation, induces Mash1, whereas a higher concentration of Aβ-(1–42) is necessary to reduce Olig2. Moreover, the induction of Mash1 may be an Aβ-specific phenomenon, whereas the reduction of Olig2 may be a common feature of the response to cell damage.

Characterization of Mash1-expressing Cells in Neuronal Culture—We first characterized the Mash1-expressing cell population in untreated neuronal cultures. Immunolabeling with anti-Mash1 antibody indicated that 7.1 ± 0.3 and 11.5 ± 0.9% of total cells expressed Mash1 in 3 and 6 DIV cultures, respectively. To define which type of cells in cerebral cortical cultures overexpress Mash1 in response to Aβ treatment, we performed double or triple immunocytochemistry on cortical cells treated with 5 μM Aβ-(1–42) for 18 h by using antibodies against Mash1 and lineage-specific markers. Most Mash1-expressing cells (about 90%) were labeled with anti-nestin, but few were labeled with anti-III-tubulin antibody in untreated cultures at 3 or 6 DIV (Fig. 2, A and B), indicating that Mash1 is not expressed in cortical neurons but rather expressed in non-neuronal dividing cells. Aβ treatment of 3 or 6 DIV cultures did not alter the proportion of III-tubulin+/Mash1+ cells (Fig. 3B). Double-immunolabeling with antibody against oligodendrocyte precursor cell marker Olig2 (Fig. 2, C and D) revealed that 70–80% of Mash1+ cells expressed Olig2 in the control medium with N2 supplement, Aβ-(1–42), R42, respectively (p < 0.001, mean ± S.E., n = 3). Thus, alteration of Mash1 and Olig2 expression by Aβ is not a nonspecific response of cells to high concentrations of non-toxic peptides. To determine whether the increase in Mash1 and the decrease in Olig2 in Aβ-treated cells depended on the concentration of Aβ or state of aggregation, we treated 3 DIV neuronal cell cultures with various concentrations of non-aggregated or aggregated Aβ-(1–42) for 18 h (Fig. 1D). We also examined whether the alteration of Mash1 and Olig2 expression is a common feature of the response of cells to damage by free radicals. An increase in the authentic Mash1 band was clearly observed when cells were treated with 1 μM non-aggregated Aβ or with 0.5–1.0 μM aggregated Aβ. The increase of the 70-kDa immunoreactive band was only detected in cultures treated with a higher concentration (5 μM) of non-aggregated or aggregated Aβ. In contrast to Aβ, 50% oxygen exposure, which induces cell death via free radicals, did not increase the total Mash1 protein level. A decrease in Olig2 expression was observed when cells were treated with 5 μM non-aggregated or aggregated Aβ or with 50% oxygen exposure. Thus, 1 μM or a higher concentration of Aβ-(1–42), independent of the state of aggregation, induces Mash1, whereas a higher concentration of Aβ-(1–42) is necessary to reduce Olig2. Moreover, the induction of Mash1 may be an Aβ-specific phenomenon, whereas the reduction of Olig2 may be a common feature of the response to cell damage.
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FIGURE 3. Aβ increased in Olig2-negative, Mash1-expressing cells in cortical culture. Three or 6 DIV cortical neurons were treated with Aβ (1–42) (final concentration 5 μM) for an additional 18 h. Cells were double-labeled with anti-Mash1 and Hoechst 33342 (A), anti-Mash1 and Tuj1 (B), or anti-Mash1 and anti-Olig2 (C), or triple-labeled with anti-Mash1, anti-GFAP, and anti-Olig2 (D). The proportion of Mash1-expressing cells in total cells (A), βIII-tubulin-expressing cells in Mash1-expressing cells (B), Olig2-negative cells in Mash1-expressing cells (C), or GFAP-expressing cells in Olig2-negative/Mash1-expressing cells (D) was estimated as indicated under "Experimental Procedures." Data are the mean ± S.E. of six experiments. ***, p < 0.001 with Student's t test compared with the untreated control.

FIGURE 4. Decrease in the number of Olig2-expressing cells in PrP-APPsw cortex. Olig2-immunolabeled cells (red) in the gray matter of the frontoparietal cortex from 15–21-month-old wild-type (A) and PrP-APPsw (B) mice. Nuclei were counterstained with Hoechst 33342 (blue). C, the number of Olig2+ cells was counted in the frontoparietal gray matter of the brain sections from wild-type (WT) and Tg2576 mice as indicated under "Experimental Procedures." Data are the mean ± S.E. (n = 3 mice per group). ***, p < 0.001 with Student's t test compared with wild-type mice.

cultures at 3 and 6 DIV. Aβ treatment increased the proportion of Olig2-negative/Mash1+ cells by 1.5-fold (Fig. 3C). The final issue we examined was whether the Mash1+/Olig2-negative cells increased by Aβ treatment were astrocytes. The percentage of cells with GFAP-positive cytoskeletons and Mash1+/Olig2-negative nuclei was very low (less than 6%) in Mash1+/Olig2-negative cells. Moreover, Aβ treatment did not increase the percentage of GFAP+ /Mash1+/Olig2-negative cells (Fig. 3D). Taken together, these findings indicated that the Mash1+/Olig2-negative cells increased by Aβ treatment were not astrocytes. Thus, Aβ(1–42) induces Mash1 expression and reduces Olig2 expression in cells without lineage-specific markers, which may possibly be immature cells, e.g., neural stem/progenitor cells.

Expression of Mash1 and Olig2 in PrP-APPsw Transgenic Mice—We employed APP mutant mice (PrP-APPsw, Tg2576) to determine whether similar alteration of Mash1 and Olig2 expression occurs in the adult neocortex. Immunolabeling with anti-Aβ revealed that 15–21-month-old heterozygous Tg2576 mice had numerous Aβ plaques in the neocortex and hippocampus. Wild-type littermates (15–21 months old) were negative for Aβ plaques (data not shown). Although no Mash1+ cells were detectable in the neocortex from adult wild-type or Tg2576 mice, Olig2+ cells were observed in the wild-type and Tg2576 neocortex (Fig. 4, A and B). The number of Olig2+ cells in the gray matter was decreased in the Tg2576 neocortex (Fig. 4C), suggesting that Aβ down-regulated Olig2 in the adult neocortex.

Overexpression of Mash1 and Depletion of Olig2 Accelerates Both Neurogenesis and Apoptosis in Neural Stem/Progenitor Cells—The above analysis showed that Aβ(1–42) alters the gene expression of basic helix-loop-helix factors, i.e., induces Mash1 and reduces Olig2 expression in immature cells, possibly neural stem/progenitor cells, in cerebral cortical cultures. We next examined whether Mash1 overexpression and Olig2 depletion in neural stem/progenitor cells promotes neurogenesis, which might contribute to preventing disease progression in AD. The neuroepithelial cells from the E15.5 telencephalon were recovered as neurospheres and maintained for 1 week. Then neurospheres were transferred to a monolayer culture in gelatin-polyornithine-coated wells and cultured in growth medium. More than 97% of the cells 1 day after dissociation were nestin+, and only a small population of cells (0.53 ± 0.14%) expressed neuronal marker βIII-tubulin, indicating that the majority of monolayer cells remained undifferentiated neural stem progenitor cells. Next, monolayer cells 1 day after dissociation were transfected with IRES-hrGFP vector alone or together with Mash1 tagged with c-Myc at the C terminus (Fig. 5A). Immunoblot analysis of neurosphere cells using antibodies against Mash1 and c-Myc 24 h after transfection confirmed the expression of exogenous Mash1 (Fig. 5B). Immunofluorescence labeling of c-Myc revealed that almost all hrGFP+ cells expressed Mash1-Myc in their nuclei 1 day after transfection (Fig. 5, C and D), but Mash1-myc+ nuclei in the hrGFP+ cells were gradually reduced (Fig. 5D), indicating the transient expression of Mash1. The transfection efficiencies of hrGFP or Mash1-hrGFP were almost the same (4.4 ± 0.9 and 3.4 ± 0.5%, respectively).
To perform Olig2 silencing experiments using specific siRNA, neurosphere cells 1 day after dissociation were transfected with three different double-stranded siRNAs for Olig2 (#18, #535, and #540) or a control scrambled siRNA (NC322) and subjected to Western blotting 2 days later to test the silencing effect of Olig2 siRNA. Among the three siRNAs for Olig2, #535 most effectively reduced Olig2 protein (Fig. 6A); however, the silencing effect of #535 assessed by Western blotting was incomplete because of low transfection efficiency. Therefore, we performed cotransfection experiments with Olig2 siRNA #535 and hrGFP vector. Immunofluorescence labeling of Olig2 revealed that almost all hrGFP + cells cotransfected with Olig2 siRNA #535 had Olig2-negative nuclei, whereas only about 10% of hrGFP + cells had Olig2-negative nuclei when cells were cotransfected with scramble siRNA (NC322) for 2 days (Fig. 6, B and C). It is unlikely that the absence of Olig2 in hrGFP + cells transfected with scramble siRNA was the result of a nonspecific silencing effect of scramble siRNA, because a similar small percentage (12.5 ± 1.9%) of Olig2-negative cells was present in the non-transfected monolayer neurosphere cultures.

To examine the effect of Mash1 overexpression under Olig2-depleted conditions on the cell fate of neural stem/progenitor cells, hrGFP or Mash1-hrGFP was cotransfected with Olig2 siRNA or scrambled siRNA into neurosphere cells. Transfected cells were cultured for 2 days and subjected to immunofluorescence for βIII-tubulin. When hrGFP alone was overexpressed, less than 5% of the hrGFP + cells were βIII-tubulin +. Overexpression of Mash1-hrGFP in the neurosphere cells increased in the population of βIII-tubulin + cells to 55% of hrGFP + cells (Fig. 7A). Suppression of Olig2 in neurosphere cells by specific siRNA also increased the population of βIII-tubulin + cells but to a lesser extent than Mash1 overexpression (Fig. 7A). However, the overexpression of Mash1 under Olig2-depleted conditions did not lead to an additional increase in βIII-tubulin + cells but, rather, led to the suppression of the increase in βIII-tubulin + cell (Fig. 7A), indicating that both Mash1 and Olig2 expression are necessary for neural differentiation. Next, to determine whether the overexpression of Mash1 under Olig2-depleted conditions induces cell death, neurosphere cells expressing hrGFP and showing DNA fragmentation were visualized with Hoechst 33342 staining (Fig. 7B). Apoptotic nuclei, which appeared in 7% of cells transfected with hrGFP alone, increased to 12% of Mash1-hrGFP + expressing cells. It is unlikely that the induction of cell death in Mash1-overexpressing neural stem cells was due to a high concentration of Mash1 in the cells because the misexpression of Mash1 in cerebral cortical neurons led to a decrease in apoptotic nuclei after withdrawal of serum (misexpression of hrGFP, 31.1 ± 1.5% cells were apoptotic; misexpression of Mash1-hrGFP, 20.2 ± 0.9% cells were apoptotic, p < 0.001; mean ± S.E., n = 4). Olig2 depletion also increased the apoptotic nuclei in cells expressing hrGFP alone and resulted in an additional increase in apoptotic nuclei in cells expressing Mash1-hrGFP. These results indicate that the overexpression of Mash1 enhances neurogenesis in

**FIGURE 5. Distribution of transfected Mash1 in neurosphere cells.** A, schematic representation of Mash1-IRES hrGFP vector. An Myc tag was attached to the C terminus of Mash1 protein. Mash1 cDNA was inserted upstream of the internal ribosomal entry site (IRES). B, Western blotting for Mash1, which was overexpressed in monolayer neurosphere cells. Cell lysates of neurosphere cells 24 h after transfection with the Mash1 construct were fractionated by SDS-PAGE on a 5–15% linear gradient gel and analyzed by immunoblotting with anti-Mash1 or anti-Myc-tag antibodies. C, distribution of Mash1 overexpressed in monosynaptic neurons. Monolayer neurosphere cells 24 h after transfection with Mash1-IRES hrGFP vector were examined for immunofluorescence of polyclonal anti-Myc-tag antibodies (red) and hrGFP (green). D, quantification of the percentage of Myc-labeled cells in hrGFP-labeled cells. The percentage of cells coexpressing Mash1-Myc and hrGFP gradually decreased during culturing, indicating that the detection of Mash1-overexpressing cells by hrGFP fluorescence is more suitable than that by Myc immunofluorescence. Data are the mean ± S.E. of three experiments.

**FIGURE 6. Reduction of Olig2 expression by RNA interference in neurosphere cells.** A, Western blotting of the nuclear fractions prepared from monolayer neurosphere cells 48 h after transfection with siRNA duplexes (20 nM). The proteins were fractionated by SDS-PAGE on a 5–15% linear gradient gel and analyzed by immunoblotting with anti-Olig2 antibodies. B, distribution of hrGFP and Olig2 in neurosphere cells. Monolayer neurosphere cells 48 h after cotransfection with hrGFP vector (3 μg/ml) and Olig2 siRNA (#535, 20 nM) or with scrambled siRNA (NC322, 20 nM) were examined for immunofluorescence of polyclonal anti-Olig2 antibodies (red) and hrGFP (green). C, quantification of the percentage of Olig2-negative cells in hrGFP-labeled cells. More than 98% of hrGFP-labeled cells were Olig2-negative when cells were cotransfected with hrGFP and Olig2 siRNA #535. Only 10% of hrGFP-labeled cells were Olig2-negative when cells were cotransfected with hrGFP and scrambled siRNA NC322, indicating a specific reduction of Olig2 in cells transfected with Olig2 siRNA #535.
Differential Regulation of Mash1 and Olig2 by β-Amyloid

Olig2-expressing neural stem/progenitor cells but accelerates the apoptosis of Olig2-depleted neural stem/progenitor cells. Thus, the induction of Mash1 and reduction of Olig2 by Aβ may promote both neurogenesis and cell death in neural stem progenitor cells.

EGF and FGF2 induce Olig2 expression (20, 21) and regulate the generation of neuronal and glial cells (22). Therefore, we examined whether the overexpression of Mash1 under EGF/FGF2-deprived conditions accelerates neurogenesis or cell death. First, we examined the effects of growth factor deprivation in neurosphere cultures on the gene expression of transcription factors involved in early neurogenesis or gliogenesis. Northern blot analysis revealed a dramatic decrease in Olig2 and increase in Mash1 in neurosphere cells 3 h after the withdrawal of EGF/FGF2 (Fig. 8A). The reduction of Olig2 continued for at least 24 h after growth factor deprivation. The level of Mash1 returned to the basal level 16 h after growth factor withdrawal. None of the transcription factors investigated here other than Olig2 was down-regulated by growth factor deprivation. Next, we examined the fate of Mash1-overexpressing cells under EGF/FGF2-deprived conditions. Neurosphere cells expressing hrGFP or Mash1-hrGFP and showing DNA fragmentation were visualized by Hoechst 33342 staining. A significantly higher level of apoptosis was found in Mash1-hrGFP+ cells cultured in the absence than in the presence of growth factors (Fig. 8B), indicating that growth factor deprivation down-regulates Olig2 expression and induces the apoptosis of Mash1-expressing cells.

DISCUSSION

Mash1 plays a role in the early steps of neurogenesis in the central nervous system (16). In addition to being strongly expressed in the early developing brain, Mash1 is also present at a low but detectable level in the cerebral cortex at the late stage of development and the adult stage (23). The up-regulation of Mash1 has been documented in the adult hippocampus after ischemia (24) and in status epilepticus (25); however, whether Mash1 is regulated in the AD hippocampus or cerebral cortex is not yet known.

Olig2 has been implicated in the specification of progenitor cells to generate neuronal subtypes (forebrain cholinergic neurons and cortical interneurons) and oligodendrocytes (26–29). An additional role of Olig2 is to maintain neural progenitor cells in the replicative state via cell cycle control (21, 30). Olig2 is widely distributed in not only the developing but also the adult brain; however, its regulation in the AD cerebral cortex remains poorly understood. The function of Olig2 in the adult cerebral cortex under pathological conditions is not yet known.

In this study we demonstrated that Aβ increases the expression of Mash1 in cerebral cortical cultures. The up-regulation of Mash1 is dose-dependent, independent of the state of Aβ aggregation, and may not be a common feature of the response to cell damage. Mash1 is expressed in nestin-positive immature cells but rarely expressed in post-mitotic neurons in cerebral cortical cultures, consistent with its expression profile in the developing ganglionic eminence and in dentate and olfactory epithelium (31–33). The majority of Mash1-positive cells in the cerebral cortical culture co-express Olig2. Despite the Mash1 mRNA and protein induction by Aβ in cerebral cortical cultures, Aβ does not increase the total number of Mash1-positive cells. Aβ increases the proportion of Olig2-negative/mash1+ cells, which are not labeled with the cell markers of neurons, astrocytes, or oligodendrocytes. The decrease in Olig2-negative/Mash1+ cells in relatively longer-term cultures suggests that Olig2-negative/Mash1+ cells may be immature cells. Thus, Aβ-(1–42) may up-regulate Mash1 and down-regulate Olig2 expression in immature cells, which are possibly neural stem/progenitor cells. Our finding of a decrease in Olig2+ cells in the neocortex from PrP-APPsw mice supports the notion that Olig2 is down-regulated by Aβ in vitro. We could not detect Mash1-immunopositive cells in the neocortex or hippocampus from adult intact or Aβ-loaded mice. This is somewhat in contrast with the findings that a low level of Mash1

FIGURE 7. Overexpression of Mash1 enhances the neural differentiation of neurosphere cells expressing Olig2 but induces the death of Olig2-depleted cells. Monolayer neurosphere cells 1 day after dissociation were cotransfected with hrGFP (3 μg/ml) and Olig2 siRNA (20 nM) or Mash1-hrGFP (3 μg/ml) and Olig2 siRNA (20 nM) and cultured in growth medium containing EGF/FGF2 for an additional 2 days. A, the number of transfected cells undergoing differentiation into neurons was counted after immunostaining for βIII-tubulin of at least 100 transfected cells for each construct in each determination. Data are the mean ± S.E. of two independent experiments with three determinations each. Results are expressed as the percentage of neurons among hrGFP-expressing cells. Statistical significance was analyzed by analysis of variance and a post hoc test; **, p < 0.001; *, p < 0.01. B, the number of transfected cells undergoing apoptosis was determined after Hoechst 33342 staining of at least 100 transfected cells for each construct in each determination. Data are the mean ± S.E. of two independent experiments with three determinations each. Results are expressed as the percentage of apoptotic cells among hrGFP-expressing cells. Statistical significance was analyzed by analysis of variance and a post hoc test; *** p < 0.0001, * p < 0.01.
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FIGURE 8. A, growth factor deprivation down-regulates Olig2 expression in neurosphere cells. Monolayer neurosphere cells were cultured in growth medium (GFs (+)) or EGF/FGF-deprived medium (GFs (−)) for 3, 16, or 24 h. One-microgram aliquots of poly(A) + RNA extracted from neurosphere cells cultured in medium with or without EGF/FGF were electrophoresed, transblotted to a nylon membrane, and hybridized with specific probes for Mash1, Olig2, Pax6, Hes1, Hes5, or Nestin mRNA. B, overexpression of Mash1 in neurosphere cells induces cell death in the absence of EGF/FGF2. Monolayer neurosphere cells 1 day after dissociation were transfected with hrGFP or Mash1-hrGFP and cultured for 1 additional day in medium with or without EGF/FGF2. The number of transfected cells undergoing apoptosis was determined after Hoechst 33342 staining of at least 100 transfected cells for each construct in each determination. Data are the mean ± S.E. of two independent experiments with three determinations each. Results are expressed as the percentage of apoptotic cells among hrGFP-expressing cells. Statistical significance was analyzed by analysis of variance and a post hoc test; ***,**, and * denote statistical significance at the 0.0001, 0.01, and 0.05 levels, respectively.

mRNA is detected in the adult cerebral cortex (23) and that up-regulation of Mash1 mRNA is found in the injured adult hippocampus (24, 25). The use of different procedures for Mash1 detection, immunofluorescence or in situ hybridization, might have caused different sensitivities of detection.

The next issue addressed here was whether Mash1 overexpression and Olig2 depletion promote or impair neurogenesis. Because it is difficult to prepare βIII-tubulin-negative/GalC-negative/GFAP-negative immature cells from neuronal cultures, we used neurosphere cells from the E15.5 day telencephalon for co-transfection experiments. We demonstrated that the overexpression of Mash1 in neurosphere cells retaining Olig2 expression induced neural differentiation rather than cell death, but Mash1 overexpression in cells in which Olig2 was depleted by specific siRNA induced cell death. Experiments manipulating the levels of EGF and FGF2 in the culture medium of neurosphere cells provided similar findings that Mash1-overexpressing cells generated more neural cells in the presence of EGF/FGF2, but they underwent death in the absence of EGF/FGF2. Supporting our findings, Olig2 together with Mash1 promotes the generation of the GABAergic neuron/oligodendrocyte lineage in the cerebral cortex (28). Thus, both Mash1 and Olig2 may be required for neurogenesis and cell survival in neural stem cells, and the down-regulation of Olig2 in Mash1-overexpressing cells may switch the cell fate from differentiation to death.

Neurogenesis has been reported to be impaired in the hippocampus in most AD mouse models (PrP-APPSW Tg, PDGF-APPV717F Tg, PS1P17L Tg, PS1M146V knockin) (6–11). However, neurogenesis in the post-mortem AD brain and in one AD mouse model (PDGF-APPsw, in Tg) was reported to be increased (4, 12). Similarly, contradictory findings have been found regarding the expression pattern of transcription factor Olig2 in AD mouse models; down-regulation of Olig2 in the cerebral cortex from PrP-APPSW mice (our study) versus up-regulation of Olig2 in the cerebral cortex from Thy1-APPPS mice (34). The increased neurogenesis and Olig2 expression appear to be restricted to transgenic mice expressing two different types of mutations in APP or APP/PS1. The transgenic makeup might influence neurogenesis or neurogenesis-related gene expression via unknown mechanisms. Neuronal loss appears to contribute to increased neurogenesis, because neocortical neuron loss is not apparent in 6–9-month-old Thy1-APPPS mice (35).

The in vitro action of Aβ on neural stem/progenitor cells is also controversial; Aβ peptide (Aβ(1–40) or Aβ(1–42)) impairs the proliferation and neuronal differentiation of cultured neural stem cells from the embryonic human telencephalon (9, 15). Conversely, Aβ promotes the neuronal differentiation of cultured neural stem cells from the newborn mouse hippocampus or adult mouse subventricular zone (13, 14). This inconsistency may not result from differences of the state of aggregation (fibril of pre-fibril) or the concentration of Aβ used in these experiments. The source of neural stem cells (human or rodent) could contribute to the conflicting findings. Human neural stem/progenitor cells might be more vulnerable to Aβ toxicity than rodent progenitors, as is true in the case of cortical neurons (36), and might fail to proliferate and differentiate in the presence of Aβ. In fact, we did not observe a decrease in nestin+ cells in rat neuronal cultures treated with Aβ for 18 h (not shown) despite the fact that the expression of Mash1 and Olig2 was altered by Aβ treatment for 18 h. Taken together with the finding that down-regulation of Olig2 impairs the proliferation of progenitors (21, 30), our data suggest that Aβ may decide the fate of progenitor cells, namely, whether to die or whether to escape from the replicative state via down-regulation of Olig2 in the rodent cortex and, consequently, may cause the failure of neurogenesis. Thus, even if Aβ induces neural stem/progenitor cells to re-express molecules critical for neurogenesis (e.g. Mash1), a reduction in other transcription factors (e.g. Olig2) that is required for cell proliferation or survival may impair neurogenesis, making it insufficient to replace neuronal loss in the AD brain. Enrichment of the brain microenvironment (20, 31), which induces Olig2 expression, may be necessary for neural stem/progenitor cells to proliferate and survive.
