The Amyloid Precursor Protein (APP) Triplicated Gene Impairs Neuronal Precursor Differentiation and Neurite Development through Two Different Domains in the Ts65Dn Mouse Model for Down Syndrome*

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Background: Individuals with Down syndrome suffer from mental retardation due to severe neurogenesis impairment.

Results: Normalization of the triplicated gene APP expression restores neuronal maturation and differentiation in trisomic NPCs, indicating that APP overexpression underpins all aspects of neurogenesis impairment. Particularly, APP overproduction contributes to neurogenesis impairment in DS.

Discussion: APP influences neuronal precursor differentiation and neurite development. We found that normalization of APP overexpression was related to increased levels within the APP/AICD system regulates neuronal differentiation and maturation in trisomic NPCs. The APP/AICD system regulates neurogenesis and neurite length through the Shh pathway, whereas the APP/secreted AP system promotes astrogliogenesis through an IL-6-associated signaling cascade. These results provide novel insight into the mechanisms underlying brain development alterations in DS.

Conclusion: APP signaling may be a target for therapeutic approaches aiming to improve brain development in DS.

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2 The abbreviations used are: DS, Down syndrome; APP, amyloid precursor protein; sAPP, secreted APP; AICD, APP intracellular domain; Shh, Sonic Hedgehog; qPCR, quantitative PCR; NPC, neuronal precursors; m.o.i., multiplicity of infection; SAG, benzo[b]thiophene-2-carboxamide, 3-chloro-N-[4-(methylamino)cyclohexyl]-N-[3-(4-pyridinyl)phenyl]methyl]- (9CI); GFAP, glial fibrillary acidic protein; ANOVA, analysis of variance; gp130, glycoprotein 130; Ptc1, patched1; Smo, Smoothened.

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This article has been withdrawn by the authors upon the request of the Journal. In December 2019, the Journal raised questions about Figs. 2 (B--D), 6A, and 7D. In particular, in Figs. 2B (Ptch1 panel) and 6A, some background features were removed. Figs. 2 (C and D) and 7D were composite images. In Fig. 2D, one of the Ptch1 bands is duplicated. The authors were able to locate most, but not all, of the original data. Inspection of the original data determined that the wrong data were selected in assembling the total STAT3 panel of Fig. 7D. The authors were willing, though, to provide alternative figures from replicated experiments and offered to repeat the experiments.

However, the Journal declined both of these offers. The authors stand by the experimental data and conclusions and state that the main results of this paper have been confirmed in the following publications: Coronel et al. (2019) Mol. Neurobiol. 56, 1248-1261; Ovchinnikov et al. (2018) Stem Cell Reports 11, 32-42; and Bailey et al. (2013) Glia 61, 1556-1569.
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The role played by different triplicated genes in brain developmental alterations in DS is scarcely elucidated. Recent findings demonstrate that the amyloid precursor protein (APP) plays a key role in normal brain development by influencing neural precursor cell proliferation, cell fate specification, and maturation (20), which suggests that triplication of this gene may compromise these processes in the DS brain. APP influences cell fate specification and neuronal maturation via two separate domains, the soluble secreted APP (sAPP) and the APP intracellular domain (AICD). sAPP promotes gliogenesis (20–22), whereas AICD negatively modulates proliferation and maturation of neural precursors (20). APP is cleaved by three types of proteases, which are designated α-, β-, and γ-secretases and which may result in functionally distinct outcomes. The non-amyloidogenic APP processing pathway involves proteolytic cleavages exerted by α- and γ-secretases resulting in the generation of N-terminal sAPPα and C-terminal fragments including P3, CTF83, and the intracellular domain (AICD). The alternative amyloidogenic APP processing pathway involves proteolytic cleavages exerted by β- and γ-secretases resulting in the generation of sAPPβ and C-terminal fragments including Aβ, CTF99, and AICD.

AICD has been shown to be involved in the transcriptional regulation of various genes (23, 24), including patched1 (Pttch1) (25). Recently, we demonstrated that neuronal precursors from the Ts65Dn mouse exhibit derangement of the Sonic Hedgehog (Shh) pathway due to an overexpression of Ptc1, the Shh pathway in vivo receptor (26). We found that Pttch1 overexpression is correlated to increased levels of AICD (26). Consequently, the proliferation defects that characterize the DS brain.

Shh signaling appears to regulate not only cell division but also neuronal precursor cell fate specification and neuronal maturation (27), suggesting that Pttch1 could contribute to derangement of the Shh pathway in the DS brain. This appears to be underlined, in addition to proliferation impairments, in the cell fate specification and neuronal maturation pathway, over increased levels of the sAPP fragment may also contribute to derangement of these processes (20) in the DS brain. The mechanisms underlying the several facets of brain development alterations in DS are largely unknown. Therefore, in the current study we exploited an in vitro model of neuronal precursor cells from the Ts65Dn mouse in order to dissect the APP-dependent molecular mechanisms underlying defective cell fate specification and neurite development.

EXPERIMENTAL PROCEDURES

Ts65Dn Mice Colony—Female Ts65Dn mice carrying a segmental trisomy of chromosome 16 (13, 28) were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained on the original genetic background by mating them to C57BL/6J (B6Eic3) F1 males. Animals were karyotyped using real-time quantitative PCR (qPCR) as previously described (29) and using PCR with primers spanning the translocation breakpoint of extra chromosome 17 (30). The animals had access to water and food ad libitum and were kept in a room with a 12:12-h dark/light cycle. Experiments were performed in accordance with the Italian and European Community law for the use of experimental animals and were approved by Bologna University Bioethical Committee. In this study all efforts were made to minimize animal suffering and to keep the number of animals used to a minimum.

NPC Cultures—Cells were isolated from the subventricular zone of newborn (postnatal day 2), euploid (n = 15), and Ts65Dn (n = 15) mice, and neurosphere cultures were obtained as previously reported (26). Cells were cultured in suspension in DMEM/F-12 (1:1) containing B27 supplements (2%), FGF-2 (20 ng/ml), EGF (20 ng/ml), heparin (5 μg/ml), and antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml). Primary neurospheres (P1) were dissociated at day 7 using Accutase (PAA, Pasching, Austria) to derive secondary neurospheres (P2). Cell cultures were kept in a 5% CO2 humidified atmosphere at 37 °C.

AICD Cloning and Lentiviral Production—cDNA encoding human AICD was generated by PCR using pGFP C1 vector (BD Biosciences) containing human AICD cDNA (31) as a template. The forward and reverse primers were as follows: AICD forward, 5'-GGGATCCATGAAGAAGAAACAGTACACAGAAGGAGGAACATGAGGAGGAAGGAACAAGAGC-3'; reverse, 5'-GGGATCCATGAAGAAGAAACAGTACACAGAAGGAGGAACATGAGGAGGAAGGAACAGAGC-3'. Replication-incompetent lentivirus was produced from HEK293FT cells using lentiviral packaging vectors pMD18T (Takara) and pCMV ΔR8.9 (Addgene) encoding human AICD were cotransfected with lentiviral packaging plasmid, pMD18T-CMVΔR8.9, pseudotyping plasmid pSVG, and lentiviral packaging plasmid. Viral supernatant was collected 48 h after transfection, filtered through a 0.45-μm low protein binding PVDF filter (Millipore), and centrifuged at 20,000 rpm (rotor type 50.2 TI) for 3 h.

In Vitro Differentiation and Treatments—Neurospheres were dissociated into a single cell suspension and plated onto poly-L-ornithine-coated 24-well chamber slides at a density of 3 × 104 cells per well. Cells were cultured for 2 days in a DMEM/F-12 medium containing EGF (20 ng/ml), FGF (20 ng/ml), and 2% FBS, and then transferred to a differentiation medium (EGF- and FGF-free plus 1% FBS) for 6 or 12 days. Every 2 days half of the medium was replaced with fresh differentiation medium. Treatments during in vitro NPC differentiation were performed as follows.

Viral Particles Transduction—NPCs were infected at day 1 post-plating with mouse APP shRNA and non-targeting shRNA control lentiviral particles (Santa Cruz Biotechnology), APP adenovirus particles (Vector Biolabs), and AICD lentiviral particles at different multiplicities of infection (m.o.i.). Twenty-four hours later the medium was replaced with a differentiation medium. We used the m.o.i. that gave an efficiency of transduction of around 50–60% at 24 h after infection. Efficiency of transduction was evaluated by infecting cells with either GFP adenovirus or GFP lentivirus particles and counting GFP-positive cells. To test whether the new differentiated neurons and astrocytes were equally infected, we evaluated the percentage of GFP-positive cells labeled with either β-tubulin III or GFAP 6.
days after infection. We found a similar percentage of new neurons and astrocytes expressing the GFP marker, indicating that there was not a lineage-related selectivity of infection.

Silencing of Ptcp1 Expression—A phosphorothioate Ptcp1 antisense oligonucleotide (26) and a scrambled oligonucleotide, as a control, were added daily for 3 days after cell plating at the final concentration of 10 \( \mu \)M.

Drugs—The following drugs were administrated on alternate days: 1 \( \mu \)g/ml anti-Alzheimer precursor protein antibody clone 22C11 (catalog, MAB348, Millipore); 250 nm benzo[b]thiophene-2-carboxamide,3-chloro-N-[4-(methylamino)cyclohexyl]-N-[(3-(4 pyridinyl)phenyl)methyl]-9CI (SAG; Enzo Life Science), 10 \( \mu \)g/ml cycloamine hydrate (Sigma), 10 \( \mu \)M N-[4-[(3,5-difluorphenacetyl)-1-allyl]-S-phenylglycine t-buty ester (Sigma).

Immunocytochemistry and Analysis of Neurite Length—For immunofluorescent staining, differentiated NPC cultures were paraformaldehyde-fixed and stained with antibodies against glial fibrillary acidic protein (1:400; GFAP mouse monoclonal, Sigma) and \( \beta \)-tubulin III (1:100; rabbit polyclonal, Sigma) or anti-oligodendrocytes (1:500; RIPC mouse monoclonal, catalog MAB1580, Millipore) as primary antibodies and with mouse FITC-conjugated (1:200; Sigma), mouse Cy3-conjugated (1:200; The Jackson Laboratory), and rabbit Cy3-conjugated (1:200; The Jackson Laboratory) as secondary antibodies. Samples were counterstained with Hoechst 33258. Ten random fields from each coverslip were photographed. The number of positive cells for each marker and total number of Hoechst-stained nuclei were counted in the areas.

Evaluation of neurite length was performed using image analysis system Image Pro Plus (Media Cybernetics, Silver Spring, MD). The average neurite length per cell was calculated by dividing the total neurite length measured from each coverslip and divided by the total number of Hoechst-stained nuclei counted in the areas.

Western Blotting—Total protein extracts from NPC cultures were obtained as previously described (26). For measurement of sAPP, the medium was collected after 6 days of differentiation. The collected conditioned medium was centrifuged at 4000 \( \times \) g for 10 min to remove the cellular debris, lyophilized, and resuspended in water plus PMSF (10 ml medium in 250 \( \mu l \) of water). Protein concentration was estimated using the Lowry method (33). An equal amount of proteins (50 \( \mu l \) from total protein extract and 300 \( \mu l \) from the medium) were subjected to electrophoresis on a 4–15% Mini-PROTEAN® TGX™ Gel (Bio-Rad) and transferred to a Hybond ECL nitrocellulose membrane (Amersham Biosciences). The following primary antibodies were used: anti-Ptcp1 rabbit polyclonal (1:500; Abcam), anti-Alzheimer precursor protein A4 clone 22C11 mouse monoclonal (catalog, MAB348 1:500; Millipore), anti p-STAT3 rabbit polyclonal (1:1000; Cell Signaling), anti-STAT3 rabbit polyclonal (1:1000; Cell Signaling), and anti-GAPDH rabbit polyclonal (1:5000; Sigma). Serial dilutions of total NPC protein extracts (10–25-50–100 \( \mu l \)) were used to determine the linear detection range of the antibodies. Densitometric analysis of digitized images was performed with Scion Image software (Scion Corporation, Frederick, MD), and intensity for each band was normalized to the intensity of the corresponding GAPDH band.

Quantitative Real Time PCR and Standard Reverse Transcription-PCR—Total RNA was isolated from differentiated NPC cultures and the hippocampus of postnatal day 2 Ts65Dn and euploid mice of either sex from the same litter using the Illustra RNAspin Mini Isolation kit (GE Healthcare) according to the manufacturer’s instructions. cDNA synthesis was achieved with 5.0 \( \mu l \) of total RNA using M-MLV Reverse Transcriptase (Promega) and oligo(dT)\(_{16}\) primers according to the manufacturer’s instructions. We used the primers that gave an efficiency that was close to 100%. Real-time PCR was performed using a GoTaq\textsuperscript{®} qPCR Master Mix kit (Promega) according to the manufacturer’s instructions in an IQ5 real time PCR detection system (Bio-Rad). The primer sequences used are as follows: peptidylprolyl isomerase A (PPIA) (NM_0008084, forward 5'-CAGTGTCGCTTTTGCAGCTTG-3’ and reverse 5'-TTTCTGCTGCTTTGGAACCTTTCCTGC-3’; GLI-Kruppel family member GLI1 (NM_010296), forward 5'-CCAGAGTCCACGGGTTTCAAGAG-3’ and reverse 5'-GTGCGCAATAGACAGAGGTAGCCGC-3’; Hairy and Enhancer of split 1 Hes1 (NM_008235.2), forward 5'-GCGTCTCCACCTTGCTCATCTTC-3’ and reverse 5’-TTTGATCCTTCAATCAGTT-3’; glial fibrillary acidic protein (GFP) (NM_213660.2, NM_011486.4), forward 5'-GCTACCCCAGCCAGTGTCAAC-3’ and reverse 5’-GCGGAGAAGCGAGGCAAC-3’; signal transducer and activator of transcription 3 Stat3 variant 1, 2, and 3 (NM_213659.2, NM_213658.2, NM_213657.2, NM_213661.2, NM_213662.2, NM_011486.4), forward 5’-GCGGAGAAGCGAGGCAAC-3’ and reverse 5’-TTTGATCCTTCAATCAGTT-3’; APP Impairs Neurogenesis in Down Syndrome

sAPP\textsubscript{x} ELISA Assay—Measurement of \( \alpha \)-secretase-cleaved sAPP\textsubscript{x} in the conditioned medium was undertaken using a sensitive and mouse/rat sAPP\textsubscript{x} kit (Demeditec Diagnostics). The assay was performed according to the manufacturer’s protocol. Briefly, 30 \( \mu l \) of conditioned medium samples were loaded in capture antibody (anti rodent APP; clone 597)-coated wells of the ELISA plate and incubated overnight at 4 °C. After several washes the wells were incubated with detection antibody (HRP-conjugated anti-rat APP; clone 18) for 30 min at 4 °C. After additional washes, chromogen solution was added to each well and kept at room temperature for 30 min. The reaction was

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stopped by adding 1 N H$_2$SO$_4$, and colorimetric signals were recorded (450 nm) using a Model 550 microplate reader (Bio-Rad). A standard curve with known amounts of recombinant rodent sAβP42 was also generated. sAβP42 values were normalized by the total protein content of the conditioned medium samples and plotted as pg/mg of protein present in conditioned medium samples.

**Statistical Analysis**—Results are presented as the means ± S.E. Statistical significance was assessed by two-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test or by the two-tailed Student’s $t$ test. A probability level of $p < 0.05$ was considered to be statistically significant.

**RESULTS**

**Neuronal Precursor Cell Cultures from the Ts65Dn Mouse Exhibit Altered Cell Fate Specification and Reduced Neurite Length, Similar to the in Vivo Condition**—We previously reported that NPCs from the subventricular zone of Ts65Dn mice exhibited a defect in cell fate specification at the third passage (P3) in culture similar to that observed in vivo (26). The number of neurons generated from trisomic NPCs was notably smaller compared with that from controls, and the number of astrocytes was larger (26). To determine whether NPCs from Ts65Dn mice exhibit changes in fate acquisition related to the passage in culture, we compared the number of neurons (β-tubulin III-positive cells), astrocytes (GFAP-positive cells), and oligodendrocytes (RIP-positive cells) produced by euploid NPCs at the first two passages (P1 and P2) versus the number of differentiated cells (number of RIP-positive cells) expected to be generated from primary neurospheres (P1) or from secondary neurospheres (P2) of euploid (EU) and Ts65Dn (TS) mice. To assess whether NPCs from Ts65Dn mice generated more oligodendrocytes than did euploid NPCs, we compared the percentages of RIP-positive cells after 6 days of differentiation (6d) or after 12 days of differentiation (12d) between NPCs derived from primary neurospheres (P1) and from secondary neurospheres (P2) of euploid (EU) and Ts65Dn (TS) mice. Values in Figure 1 are the means ± S.E. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). These results indicate that cultures of differentiated NPCs are a suitable model for studying the mechanisms underlying neurogenesis impairment in DS because they exhibit impaired acquisition of a neuronal phenotype and neuronal maturation, similar to the in vivo condition (5, 17–19, 32, 36).
high number of differentiated cells at P1, we used P1 cultures for the further steps of our study.

APP/AICD-dependent Ptc1 Overexpression in Differentiated Neuronal Precursor Cell Cultures from the Ts65Dn Mouse—

We have recently demonstrated that neuronal precursors from the Ts65Dn mouse exhibited Shh pathway derangement due to overexpression of Ptc1 (26). Ptc1 overexpression was due to increased levels of AICD, a transcription-promoting fragment of the trisomic gene APP. We first sought to establish whether these defects are retained in differentiated trisomic NPC cultures. We found that trisomic cultures exhibited overexpression of APP and Ptc1, both at RNA and protein levels (Fig. 2, A and B). To verify whether Ptc1 overexpression in trisomic cultures was APP-dependent, we reduced APP expression by using lentiviral-mediated RNA interference. At increasing m.o.i. (0.5, 2.5, and 5), APP expression in trisomic NPCs was progressively reduced (Fig. 2C). In parallel with the reduction in APP expression, there was a reduction in Ptc1 protein level (Fig. 2C). In contrast, in euploid NPCs a reduction in APP expression through RNA interference (m.o.i. 5.0) was not accompanied by a concomitant reduction in Ptc1 expression (Fig. 2D). This is in line with previous evidence showing that in euploid neurospheres AICD does not bind to the Ptc1 promoter (Trazzi et al. (26)), suggesting that AICD can modulate Ptc1 expression only when overexpressed. We next examined the effects of increased levels of APP on Ptc1 expression in euploid NPCs. After infection with recombinant adenovirus particles (m.o.i. 25) or AICD lentiviral particles, to inhibit AICD (26), we found that this treatment down-regulated Ptc1 expression in trisomic NPCs (Fig. 2D), whereas, as expected (see above), it had no effect on Ptc1 expression in euploid NPCs (Fig. 2D). AICD overexpression increased Ptc1 expression in euploid NPCs (Fig. 2D), confirming the relationship between AICD levels and Ptc1 expression in trisomic NPCs.

Normalization of APP Expression in Neuronal Precursor Cell Cultures from the Ts65Dn Mouse Restores Cell Fate Specification and Neurite Length—The finding that APP (and Ptc1) overexpression is retained in differentiated trisomic cells supports the hypothesis that the defective differentiation of trisomic NPCs may be APP-dependent. We found that in trisomic NPC cultures, APP interference treatments significantly increased the number of neurons and reduced the number of astrocytes compared with untreated trisomic cultures (Fig. 3, A and C). At m.o.i. 2.5, the multiplicity of infection at which APP and Ptc1 expression in trisomic NPCs became similar to that of the euploid NPCs (Fig. 2C), cell fate specification of trisomic NPCs completely recovered (Fig. 3, A and C). APP overexpression in euploid cultures was accompanied by a reduction in the number of neurons and an increase in the number of astrocytes (Fig. 3, A and C). Taken together these data strongly suggest that APP overexpression underlies the aberrant cell fate specification that characterizes trisomic NPCs.

To assess whether the APP-dependent defective differentiation of trisomic NPCs was due to AICD accumulation, we overexpressed AICD in euploid cultures. We found that AICD overexpression significantly decreased the number of neurons

Figure 2. Ptc1 overexpression is mediated by the APP/AICD system in differentiated NPCs from Ts65Dn mice. A, shown is relative quantification by RT-qPCR of APP and Ptc1 expression in 6-day-differentiated NPCs derived from Ts65Dn (T5, n = 6) and euploid (E, n = 6) mice, given as a percentage of the euploid condition. Values represent the mean ± S.E. *p < 0.05; **, p < 0.001 as compared with the euploid untreated condition (100%). Values are the means ± S.E. **, p < 0.01 as compared with untreated trisomic samples (Bonferroni test after ANOVA). B, shown is the effect of APP silencing on APP and Ptc1 expression in differentiating NPCs from Ts65Dn mice. C, shown is the effect of APP silencing on APP and Ptc1 expression in differentiating NPCs from Ts65Dn mice. NPCs from Ts65Dn mice (n = 5) were infected with APP shRNA lentiviral particles at increasing m.o.i. (0.5, 2.5, and 5.0). NPCs from euploid mice (n = 6) were used as a control. APP and Ptc1 protein levels were normalized by GAPDH content and are expressed as percentages of the euploid untreated condition (100%). Values are the means ± S.E. **, p < 0.01; ***, p < 0.001 as compared with the euploid condition; #, p < 0.05; ##, p < 0.01 as compared with untreated trisomic samples (Bonferroni test after ANOVA). D, shown is the effect of APP or AICD overexpression and APP or AICD down-regulation on APP and Ptc1 expression in differentiating NPCs from euploid and Ts65Dn mice. NPCs from euploid mice (n = 5) were infected with APP shRNA lentiviral particles (m.o.i. 5) or APP adenosine particles (m.o.i. 25) or AICD lentiviral particles. To inhibit AICD formation, NPCs from euploid (n = 4) and Ts65Dn (n = 6) mice were treated with the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyloxycarbonyl ester (DAPT, 10 μM). APP and Ptc1 protein levels were normalized by GAPDH content and are expressed as percentages of the euploid untreated condition (100%). Values are the means ± S.E., **, p < 0.01; ***, p < 0.001 as compared with the euploid condition; #, p < 0.05 as compared with untreated trisomic samples (Bonferroni test after ANOVA).
compared with untreated euploid cultures (Fig. 3, A and C). In contrast, the number of astrocytes did not undergo an increase (Fig. 3, A and C), suggesting that AICD/Ptc1 signaling in trisomic NPCs is responsible for the reduced neuorgenosis but does not underpin increased astrogliogenesis.

We next determined whether impaired neurite outgrowth in trisomic NPCs was also dependent on overexpression of the APP system. The addition of m.o.i. 0.5 and 2.5 APP shRNA lentiviral particles to trisomic NPC cultures induced a dose-dependent increase in neurite outgrowth (Fig. 3, B and D). Restoration of APP expression in trisomic NPCs (m.o.i. 2.5) brought about a complete recovery of neurite outgrowth (Fig. 3, B and D). Conversely, APP as well as AICD overexpression reduced neurite outgrowth in euploid cultures (Fig. 3, B and D), which is in line with the reduced neurite length found in trisomic NPCs.

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**FIGURE 3. Silencing of APP restores cell fate specification and morphology of NPCs from Ts65Dn mice.** Trisomic NPCs were infected with APP adenovirus particles (m.o.i. 25) or AICD lentiviral particles. APP shRNA lentiviral particles at an m.o.i. of 0.5 and 2.5. Euploid NPCs were morphologically normal (A) and differentiated into NPCs from Ts65Dn mice.

**FIGURE 4. Shh Pathway Activation in Neuronal Precursor Cell Cultures from the Ts65Dn Mouse Restores Neurogenesis and Neurite Length but Not Astrogliogenesis**—To determine whether derangement of the Shh pathway due to Ptc1 up-regulation underlies impaired cell fate specification in trisomic NPCs, we treated NPCs with SAG, an agonist for Smoothened (Smo) that relieves the inhibitory effect of Ptc1 on Smo (37) (Fig. 4C). Although increased activation of the Shh pathway by SAG had no effect on the percentage of new neurons and astrocytes in euploid NPCs (Fig. 4A), in treated trisomic NPCs the percentage of new neurons was strongly enhanced (Fig. 4B), in treated trisomic NPCs the percentage of new neurons was strongly enhanced (Fig. 4B), in treated trisomic NPCs the percentage of new neurons was strongly enhanced (Fig. 4B), in treated trisomic NPCs the percentage of new neurons was strongly enhanced (Fig. 4B).

Although increased activation of the Shh pathway by SAG had no effect on the percentage of new neurons and astrocytes in euploid NPCs (Fig. 4A), in treated trisomic NPCs the percentage of new neurons was strongly enhanced (Fig. 4B).
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The Gli transcription factors are mediators of Shh signaling and are transcriptionally regulated by it. Several studies have demonstrated that all Gli proteins have neurogenic properties (40–42). We found that Gli1 and Gli2 were down-regulated (-66% and -40%; p = 0.0029 and p = 0.00029, respectively) in differentiated trisomic NPCs whereas no differences in Gli3 expression were found between euploid and trisomic cultures (Fig. 5A). Of the three family members, Gli2 had the strongest neurogenic properties (42, 43). Treatment with SAG increased Gli1 expression but had no effect on that of Gli2 (Fig. 5A). In contrast, in control cultures SAG treatment increased Gli1 expression but had no effect on that of Gli2 (Fig. 5A). Consistent with the absence of effects on Gli2 expression, the expression of Mash1 was down-regulated (−30%; p = 0.012, t test) in trisomic NPCs and that SAG treatment restored its expression (Fig. 5B). Consistent with the absence of effects on Gli2 expression, the expression of Mash1 was unaffected by SAG treatment in control NPC cultures (Fig. 5B). Looking at the expression of the astrocytic gene GFAP, we found that, consistent with immunohistochemistry data (Fig. 1A), GFAP expression was up-regulated in trisomic NPC cultures and that SAG treatment did not influence its expression (Fig. 5B).

Taken together, these results suggest that in trisomic NPCs, Shh pathway inhibition due to Ptc1 up-regulation impairs neurogenesis and neurite length but not astrogliogenesis. This implies that APP overexpression affects astrogliogenesis through a different pathway(s).

Increased Levels of sAPP in Neuronal Precursor Cell Cultures from the Ts65Dn Mouse Increase Astrogliogenesis—It has been shown that treatment of NPCs with either sAPPα or sAPPβ increases the number of cells expressing the astrocytic marker GFAP (21, 22). This suggests that increased levels of sAPP, due to the tripping of APP, may be responsible for the increased astrogliogenesis that characterizes the trisomic condition. We first examined the levels of sAPP in the culture medium of euploid and trisomic differentiated cultures. We found that sAPP levels were notably higher in the medium of trisomic cultures than in that of euploid cultures (Fig. 6, A and B). To clear the effects of increased sAPP levels, we treated trisomic NPC cultures with an antibody (22C11) that, by cross-linking APP at the N-terminal domain (i.e. the domain belonging to sAPP), prevents the interaction of sAPP with numerous cell surface receptors (44). Treatment with increasing 22C11 concentrations proportionally reduced the number of GFAP-positive cells in trisomic NPC cultures, and at high doses it normalized the number of GFAP-positive cells to euploid levels (Fig. 6C). No effect was found on the number of neurons (Fig. 6C). To confirm that the increased astrogliogenesis in trisomic NPCs was due to increased sAPP levels and not to the APP/AICD/Shh system, we inhibited APP expression using APP shRNA treatment. We concomitantly inhibited the Shh pathway (using cyclopamine) to prevent activation of the Shh pathway due to the reduction of AICD levels. We found that treated trisomic NPC cultures underwent a decrease in the number of astrocytes with no changes in the number of neurons compared with untreated trisomic cultures (Fig. 6C). These findings demonstrate that increased levels of sAPP in

overexpression. To gain further evidence that in trisomic NPCs the Shh pathway is functionally inactive due to Ptc1 up-regulation, we reduced Ptc1 expression by using an antisense oligonucleotide that restores Ptc1 overexpression in trisomic NPCs to euploid levels (26). We found that although antisense treatment restored the number of new neurons in trisomic NPCs, with no effect on the number of astrocytes (Fig. 4A), it had no effect on the proportion of new neurons and astrocytes in euploid NPCs (Fig. 4A). In view of the role of the Shh pathway in neuronal maturation (38, 39), we sought to determine whether Ptc1 up-regulation also underlies reduced neurite length of trisomic NPCs. We measured neurite length of the new neurons after either activation of the Shh pathway by SAG or inhibition by cyclopamine. Although in euploid NPC cultures treatment with SAG had no effect on neurite length (Fig. 4B), in treated trisomic NPC cultures the neurite length of the new-generated neurons underwent a large increase (Fig. 4B). Conversely, inhibition of the Shh pathway by cyclopamine reduced neurite length in euploid neurons but had no effect on trisomic neurons (Fig. 4B). Although treatment with the antisense for Ptc1 completely restored neurite length in trisomic neurons (Fig. 4B), it had no effect on neurite length in euploid neurons (Fig. 4B).

The Gli transcription factors are mediators of Shh signaling and are transcriptionally regulated by it. Several studies have demonstrated that all Gli proteins have neurogenic properties (40–42). We found that Gli1 and Gli2 were down-regulated (-66% and -40%; p = 0.0029 and p = 0.00029, respectively) in differentiated trisomic NPCs whereas no differences in Gli3 expression were found between euploid and trisomic cultures (data not shown). In trisomic cultures Gli1 and Gli2 expression was increased by SAG treatment (Fig. 5A). In contrast, in control cultures SAG treatment with SAG had no effect on that of Gli2 (Fig. 5A). Of the three family members, Gli2 had the strongest neurogenic properties (42, 43). Treatment with increasing 22C11 concentrations proportionally reduced the number of GFAP-positive cells in trisomic NPC cultures, and at high doses it normalized the number of GFAP-positive cells to euploid levels (Fig. 6C). No effect was found on the number of neurons (Fig. 6C). To confirm that the increased astrogliogenesis in trisomic NPCs was due to increased sAPP levels and not to the APP/AICD/Shh system, we inhibited APP expression using APP shRNA treatment. We concomitantly inhibited the Shh pathway (using cyclopamine) to prevent activation of the Shh pathway due to the reduction of AICD levels. We found that treated trisomic NPC cultures underwent a decrease in the number of astrocytes with no changes in the number of neurons compared with untreated trisomic cultures (Fig. 6C). These findings demonstrate that increased levels of sAPP in
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trisomic NPCs specifically increase astrogliogenesis without influencing neuronogenesis.

To determine whether the reduced neurite length of trisomic neurons was due to high sAPP levels, we treated trisomic NPC cultures with either increasing 22C11 concentrations or with APP shRNA plus cyclopamine. A comparison of treated with untreated trisomic NPC cultures showed no effect on neurite length (Fig. 6D), indicating that the APP/sAPP system does not impair neurite development.

The structural property of the N-terminal domain of sAPPα indicates that secreted soluble APP may have a growth factor-like function by interacting with cell surface receptors (45). It has recently been shown that sAPPα overexpression may result in enhancement of the Notch pathway (46) as well as of the IL-6 cytokine-related pathway (47), which are crucial for the acquisition of a glial phenotype (48, 49). To identify the mechanism(s) by which sAPP increases astrogliogenesis in trisomic NPCs, we examined gene expression of various molecules downstream from the Notch and IL-6 cytokine-related pathways. We found no differences between trisomic and euploid NPC cultures in the expression of Hes1 (Fig. 7A), the sAPP-deletion product of APP (Fig. 7B), and p-STAT3 levels (Fig. 7C), shown is a Western blot analysis of p-STAT3 expression in cultures from Ts65Dn (n = 4) and euploid (EU, n = 4) mice. Trisomic NPC cultures were treated with the antibody 22C11 (doses, 0.5 and 1.0 μg/ml). Euploid NPCs were infected with APP adenovirus particles (m.o.i. 25). p-STAT3 levels were normalized to total STAT3 content. Data in A–D are given as a percentage of the euploid untreated condition. Values represent the mean ± S.E. *, p < 0.05; **, p < 0.01; ****, p < 0.001 (Bonferroni test after ANOVA).

The APP/sAPP system mediates the increased astrogliogenesis in trisomic NPCs specifically increases astrogliogenesis without influencing neuronogenesis.
Members of the IL-6 family of cytokines activate the signal-transducing receptor protein, gp130, and induce phosphorylation of Janus kinases (JAK) followed by activation of signal transducers and activators of transcription (STAT1 and STAT3). Then phosphorylated STAT3 translocates into the nucleus and stimulates target gene expression by interacting with the STAT3 binding element of target genes, including GFAP (see Fig. 9). JAK1 and gp130 belong to the transcriptional targets of STAT3, and consequently, the IL-6 cytokine-related pathway is efficiently modulated by an auto-regulatory positive loop (47). We found that although STAT3 gene expression levels were similar in trisomic and control NPC cultures (Fig. 7B), gp130 and JAK1 levels were significantly up-regulated in trisomic cultures (Fig. 7C), indicating an increased IL-6 cytokine-related pathway activation in trisomic NPCs. To determine whether the increased activation of the IL-6 cytokine-related pathway in trisomic NPCs was dependent on increased levels of sAPP, we treated trisomic NPC cultures with the 22C11 antibody to inhibit sAPP interaction with target receptors. We found that treatment with 22C11 drastically reduced the expression of gp130 and JAK1 as well as that of GFAP (Fig. 7, B and C). The phosphorylation status of STAT3 is known to be crucial for its function (50). Although we did not observe a difference in the gene expression level of STAT3, we found that in trisomic NPCs the phosphorylation status of STAT3 was increased in comparison with euploid NPCs. Treatment with the 22C11 antibody dose-dependently suppressed p-STAT3 expression (Fig. 7D). A similar increase in STAT3 phosphorylation was observed in euploid NPCs infected with APP, suggesting that the ensemble of these data suggest that APP overexpression hyperactivate the IL-6 cytokine-related pathway and increasing astrogliogenesis.

Inhibition of the Shh Pathway—The APP/AICD-dependent Alteration of the Shh Pathway Impairs Neurogenesis in Down Syndrome

We have previously reported that increased levels of AICD derange the Shh pathway in trisomic NPCs (26). We found here that Ptc1 up-regulation is also present in differentiated trisomic NPCs due to AICD overexpression and that the AICD/Ptc1 system negatively influences neurogenesis and neurite outgrowth by inhibiting the Shh pathway. Importantly, Shh pathway activation, induced either by SAG or by the silencing of Ptc1 expression, restores neurogenesis and neurite length but not astrogliogenesis. These results indicate that the Shh pathway selectively promotes neural progenitor cell differentiation into neurons. This is in agreement with a recent study showing that Shh activation, through carboxylated erythropoietin (CEPO), specifically enhances neurogenesis and promotes neurite outgrowth (51).

Shh pathway activation leads to the breakdown of a large protein complex formed by Fused, Sufu, and Glis in the cytoplasm and releases the Gli transcription factors. The released
Glis translocate into the nucleus, resulting in transcriptional activation of specific target genes, including the Glis themselves. Gli proteins (Gli1, Gli2, and Gli3) were recently shown to be involved during neurogenesis in vivo, creating a dynamic physical network (41). Of the three family members, Gli2 had the strongest neurogenic properties (42, 52). In particular, it has been shown that overexpression of Gli2 induces neuronogenesis, but not gliogenesis in P19 EC cells, and increases the expression of the pro-neurogenic gene Mash1 (43). We found here that two of the Glis proteins (Gli1 and Gli2) were down-regulated in differentiated trisomic NPCs and that treatment with an agonist of the Shh pathway (SAG) increased Gli1 and Gli2 expression. Evidence from microarray database analysis through the web-based NextBio™ software shows down-regulation of Gli2 in trisomy 21 and the Ts65Dn model, suggesting the involvement of this gene in the aberrant DS phenotype. Consistent with this hypothesis, our data suggest that down-regulation of Gli2 in trisomic NPCs may underlie neuronogenesis impairment. The finding that after Shh pathway activation Gli1 expression increased in control cultures without a concomitant increase in neurogenesis makes it unlikely that Gli1 is responsible for neuronogenesis reduction in trisomic cells.

Pro-neuronal basic helix-loop-helix transcription factors, including Mash1, promote neural progenitor cell differentiation into neurons (53). The present study shows that in trisomic NPCs, impairment of the Shh pathway was associated with down-regulation of Mash1 expression and that a Shh signaling pathway restored Mash1 expression. These findings suggest that a reduced expression of endogenous Mash1 may underlie defective acquisition of a neuronal phenotype and impairment of neurite development in trisomic NPCs. This is in agreement with a recent study showing that exogenous sAPP induces glial differentiation in the human embryonal carcinoma cell line NT-2/D1 via the Notch signaling pathway (44). However, we could not find an sAPP-dependent neuronal differentiation. This discrepancy may be due either to the different cellular system used, i.e., Mash1, or to differences in the DS phenotype, suggesting that a specific role of sAPP as a gliogenic factor exists. The astrocyte differentiation process of NPCs appears to be tightly modulated by various extrinsic (e.g., leukemia inhibitory factor, ciliary neurotrophic factor, Notch, and bone morphogenetic proteins) and intrinsic (e.g., Ngln1, Ngln2, and DNA methylation) factors. A recent study has revealed that the IL-6/gp130 signaling pathway plays a pivotal role in astrocyte differentiation of NPCs (47). Based on this evidence, it has been postulated that sAPP may affect the cell fate of neural precursor cells in DS via the activation of the IL-6/gp130 signaling pathway (47). Our data confirm this suggestion, showing that increased levels of APP/sAPP in trisomic NPCs enhance the acquisition of an astrocytic phenotype via the activation of the IL-6/gp130 signaling pathway. Cross-talk of the Notch and JAK/STAT pathways in the induction of glial differentiation in NPCs through physical interaction between Hes1 and JAK2 has recently been reported (44, 58). It has been reported that treatment with exogenous sAPP induces glial differentiation of the human embryonal carcinoma cell line NT-2/D1 via the Notch signaling pathway (44). However, we did not find an sAPP-dependent Hes1 up-regulation in trisomic NPCs, suggesting that activation of the Notch signaling pathway is not involved in sAPP-induced glial differentiation in NPCs. This discrepancy may reflect differences in the cellular system used, i.e., Mash1, or to differences in the DS phenotype, suggesting that a specific role of sAPP as a gliogenic factor exists.

The APP/sAPP-dependent IL-6/Cytokine-related Pathway Impairs Astrogliogenesis in Trisomic NPCs—The possibility that increased Glis expression is due to APP overexpression in DS is supported by recent evidence showing that human neuronal precursors overexpressing APP exhibit increased GFAP and decreased MAP2 expression (54). APP is a very complex molecule that is the source of numerous fragments with varying effects during brain development. In non-pathological situations, APP is predominantly cleaved by the α-secretase within the β-amyloid sequence to release a soluble form of APP, sAPP. sAPP is normally present in brain tissues and circulates in the cerebrospinal fluid (55). A number of in vitro studies have attempted to shed light on the physiological functions of sAPP in the brain, showing that sAPP enhances synaptogenesis, neurite outgrowth, cell survival, adhesion, and proliferation of embryonic neural stem cells (56, 57). In addition, recent in vitro and in vivo evidence shows that sAPP administration promotes astrogliogenesis (21, 22, 47). Our findings suggest that high levels of sAPP or cell surface-associated APP have a role in the increased astrocytic differentiation that characterizes trisomic NPCs. We found no effect of the APP/sAPP system on neuronogenesis or neurite length in trisomic NPCs, suggesting a specific role of sAPP as a gliogenic factor.
However, the Ts1Cje mouse shows spine and connectivity alterations (60) and reduction in cerebellar granule cell number (61) less severe than the Ts65Dn mouse. Interestingly a high resolution analysis of human segmental trisomies suggests that more than one mental retardation critical region of HSA21 exists (62). Thus, many different triplicated genes are likely to contribute to the brain and behavioral phenotype in DS. Triplication of different genes may culminate into similar effects by acting on multiple, intersecting/converging pathways. This may explain, for instance, why APP triplication affects but is not essential for some of the trisomic phenotypes. The development of a new mouse strain obtained by crossing APP transgenic with Ts1Cje mice may provide additional clues as to the role of APP on brain phenotype in the trisomic condition.

Based on our present and previous findings we propose a model for the APP-mediated developmental defects in the Ts65Dn mouse (Fig. 9). These findings indicate that a common molecular denominator may be involved in the impairment of cell proliferation, cell fate specification, and neuronal matura-

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FIGURE 9. Alterations of APP-modulated pathways in neuronal precursor cells from Ts65Dn mice. The diagram shows APP triplication-dependent derangement of two key pathways involved in proliferation, cell fate specification, and neurite development. Excess of APP/sAPP in trisomic NPCs over-
stimulates the IL-6 pathway, thereby inducing GFAP overexpression and enhancing astrogliogenesis. On the other hand, Ptch1 overexpression, induced by APP-derived AICD, inhibits the Shh pathway and results in down-regulation of the transcription factor Mash1, thereby reducing proliferation, neuronogenesis, and neurite development. sAPP, secreted N-terminal soluble fragment of APP.
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