Dynamic Regulation of Expression and Phosphorylation of Tau by Fibroblast Growth Factor-2 In Neural Progenitor Cells from Adult Rat Hippocampus

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The nature of the extracellular signals that regulate the expression and the phosphorylation of the microtubule-associated protein tau, which is aberrantly hyperphosphorylated in Alzheimer disease and other adult-onset neurodegenerative diseases, is not known. We have found that neural progenitor cells from adult rat hippocampus express adult isoforms of tau and that the expression and the phosphorylation of tau are regulated by fibroblast growth factor-2 (FGF-2). Astrocytes that are differentiated from these cells by stimulation with ciliary neurotrophic factor express phosphorylated tau similarly when cultured in the presence of FGF-2. In fetal progenitor cells that express only the fetal tau isoform, expression, but not the phosphorylation, of this protein is regulated by FGF-2 in cultures of higher passages. The FGF-2-mediated tau hyperphosphorylation is inhibited by lithium, an inhibitor of glycogen synthase kinase-3 (GSK-3), but not by inhibitors of mitogen-activated protein kinase or the cyclin-dependent kinases. Furthermore, both GSK-3 activity and the phosphorylation of tau increase when the concentration of FGF-2 is increased up to 40 ng/ml. These results demonstrate that proliferating adult rat hippocampal progenitor cells express adult isoforms of tau stably and that FGF-2 upregulates the expression and, by upregulating GSK-3 activity, the phosphorylation of tau.

Key words: tau; fibroblast growth factor-2; glycogen synthase kinase-3; neural progenitor cells; phosphorylation; adult tau isoforms; Alzheimer Disease

Neural progenitor (or stem) cells are multipotential precursor cells that can give rise to both neurons and glia in the fetal and adult CNS (for review, see McKay, 1997). Most of the progenitor cells from adult hippocampus expanded by fibroblast growth factor-2 (FGF-2) are immunohistochemically positive for the intermediate filament nestin, the neuronal marker microtubule-associated protein-2C (MAP-2C), neuron-specific enolase, and the immature glial marker O4, but only a few of the cells have been found to be positive for markers of differentiation such as glial fibrillary acidic protein (GFAP), myelin basic protein, or neurofilament H (Gage et al., 1995; Palmer et al., 1997). However, little is known about the expression of the neuronal MAP tau in these progenitor cells. The expression of tau is regulated developmentally; i.e., whereas in adult mammalian brain several isoforms are produced from a single gene by alternative splicing, in fetal brain only a single isoform is expressed that corresponds to the smallest of the tau isoforms (Lee et al., 1988; Goedert et al., 1989; Kosik et al., 1989). One of the most important post-translational modifications of tau is phosphorylation, because the degree of phosphorylation regulates its biological activity (Lindwall and Cole, 1984). Normal adult tau, which contains two to three phosphate groups, promotes the assembly of microtubules *in vitro*, and fetal tau, which is hyperphosphorylated to an intermediate degree, has less activity (Yoshida and Ihara, 1993). In Alzheimer disease (AD), tau is hyperphosphorylated abnormally (Grundke-Iqbal et al., 1986a,b; Iqbal et al., 1986). It contains up to 9 mol of phosphate per mole of the protein (Köpke et al., 1993) and is microtubule assembly-incompetent (Alonso et al., 1994; Iqbal et al., 1994).

In the present study, using adult hippocampal progenitor cells expanded by FGF-2, we demonstrate for the first time that these cells express adult isoforms of tau that are phosphorylated, especially at Ser 195/198/199/202, the Tau-1 site. Interestingly, in these cells, FGF-2 upregulates the expression and phosphorylation of tau at the Tau-1 site. We show that the mechanism of the hyperphosphorylation of tau by FGF-2 involves the upregulation of the glycogen synthase kinase-3 (GSK-3) activity; the mitogen-activated protein kinase (MAPK) and cyclin-dependent kinase (cdk) pathways are not involved in this mechanism. On the other hand, fetal hippocampal progenitor cells expanded by FGF-2 express only the fetal isoform of tau for which the expression is regulated by FGF-2, but only in the late passages, and FGF-2 has no effect on its phosphorylation. Thus, FGF-2 is the first identified extracellular regulator for expression and phosphorylation of tau in neural cells derived from adult CNS.

MATERIALS AND METHODS

Antibodies, enzyme inhibitors, isolation of tau. The following phosphorylation-dependent, site-specific monoclonal and phosphorylation-independent polyclonal antibodies to tau were used: Tau-1 (to unphos-

phorylated Ser 195, 198, 199, or 202; numbers according to the longest human tau isoform, corresponding to Ser 186, 189, 190, or 193 in the longest rat tau isoform; ascites, 1:50,000) (Binder et al., 1985; Grundke-Iqbal et al., 1986a; Szendrei et al., 1993); PHF-1 (to phosphorylated Ser 396/404 in human and Ser 387/395 in rat; culture supernatant, 1:100) (Greenberg et al., 1992; Otvos et al., 1994); M4 (to phosphorylated Thr 231/Ser 235 in human and Thr 222/Ser 226 in rat; ascites, 1:2000) (Hasegawa et al., 1993); 12E8 (to phosphorylated Ser 262/356 in human and Ser 253/347 in rat; 1 μg/ml) (Seubert et al., 1995); rabbit antisera to isolated bovine tau, 92e (1:5000; Grundke-Iqbal et al., 1988); and to recombinant human tau $_{410}$, 134d [1:5000; raised according to the method in Grundke-Iqbal et al. (1988)]. Other primary antibodies used were monoclonal antibody SMI 33 to dephosphorylated neurofilaments H and M (1:1000; Sternberger Monoclonals, Baltimore, MD); monoclonal antibody DM1A to α-tubulin (1:2000; Sigma, St. Louis, MO); polyclonal antibody 972 to dephosphorylated MAP-2A–C (1:10,000; Sánchez et al., 1995); monoclonal and polyclonal antibodies to GFAP, mono-GFAP (1:1000; Boehringer Mannheim, Indianapolis, IN), poly-GFAP (1:2000; Dako, Carpinteria, CA), monoclonal antibodies to nestin, Rat-401 (1:1000; PharMingen, San Diego, CA), and rabbit antiserum to nestin #130 (1:1000; Tohyama et al., 1992); and rabbit antiserum R133d to GSK-3 (1:500; Pei et al., 1997). The secondary antibodies used were goat anti-mouse IgG (1:100), followed by Clono PAP (1:100), both purchased from Sternberger (Jarrettsville, MD); ^{125}I-conjugated anti-mouse and anti-rabbit IgG antibodies (0.1 μg/ml; Amersham, Arlington Heights, IL) for radio immunoblots; Oregon Green 488-labeled goat anti-mouse (1:1000; Molecular Probes, Eugene, OR) and Texas Red-labeled goat anti-rabbit IgG antibodies (1:2000; Molecular Probes) for immunocytochemistry. PD 98059, butyrolactone I, and LiCl were purchased from Calbiochem (La Jolla, CA), Biomol (Plymouth Meeting, PA), and Sigma, respectively. Recombinant human taus, tau$_{410}$ (tau 39, three-repeat tau with two N-terminal inserts), tau$_{383}$ (tau 24, four-repeat tau without N-terminal inserts), and tau$_{352}$ (tau 23, three-repeat tau without N-terminal inserts) were prepared as previously described (Singh et al., 1995b). Hyperphosphorylated tau (AD P-tau) was purified from Alzheimer brain as described previously (Köpke et al., 1993).

Isolation of adult hippocampal progenitor cells. Approximately 3-month-old rats were killed by lethal injection of Nembutal (200 mg/kg body weight; Abbott Laboratories, North Chicago, IL); the hippocampus was dissected, and the cells were dissociated according to the slightly modified method of Brewer (1997). Dissected hippocampus was cut into small ($\sim$0.5 mm^3) pieces with a razor blade in Hibernate A containing 2% B27 supplement and 0.5 mM glutamine at 4°C (all of which were purchased from Life Technologies, Grand Island, NY). The tissue pieces were digested with 2 mg/ml papain (Worthington, Freehold, NJ) in Hibernate A/B27 for 30 min at 30°C with shaking on a reciprocal shaker at 170 rpm, washed gently with warm Hibernate A/B27 once, and dissociated in three 2 ml volumes of Hibernate A/B27 by brief mechanical trituration with a siliconized Pasteur pipette. To remove debris, we layered the resulting 6 ml of cell suspension over a 4 ml step density gradient (Optiprep, Life Technologies; at 7, 9.4, 11.7, and 16.4%, 1 ml each, in Hibernate A/B27) and centrifuged it at 800 × *g* at room temperature for 15 min. The top 7 ml of the supernatant that contained only debris was discarded, and the remaining 3 ml of supernatant, including the dense band of cells and some debris and the pellet, were collected together and diluted in 5 ml of Hibernate A/B27. After 5 min of centrifugation at 800 × *g*, the cell pellet was resuspended in Neurobasal A (12.5 mM NaCl plus Neurobasal; Life Technologies) containing 2% B27 supplement and 0.5 mM glutamine, and 1 × 10^6 of cells were plated onto glass slides precoated with 0.01% poly-L-lysine (135 kDa; Sigma). Cultures were incubated at 37°C with 5% CO$_2$ for 1 hr. Then the slides were washed once with warm Neurobasal A/B27 to remove debris and were cultured with Neurobasal A/B27 containing 0.5 mM glutamine, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 5 ng/ml FGF-2 (Life Technologies). Half of the medium was changed to new medium with a double amount of fresh FGF-2 every 3–4 d, and the cells were passaged at 70–90% confluency by incubating them briefly in warm PBS and scraping them with a cell scraper. After the third passage the plates were precoated with 1 μg/ml of fibronectin (Life Technologies) in addition to 0.01% poly-L-lysine; the FGF-2 concentration in the medium was increased to 10 ng/ml. Cultures from the fifth to eleventh passages were used for this study.

Isolation of fetal hippocampal progenitor cells. Fetal hippocampal progenitor cells were isolated and expanded according to the slightly modified method of Johe et al. (1996). Briefly, rat fetal hippocampus (gesta-

tion day 16; day of conception is day 1) was dissected in Hibernate A/B27 and dissociated by brief trituration in the same medium. The cells were collected by centrifugation as above and resuspended in Neurobasal containing 2% B27 supplement, 0.5 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml FGF-2. Cells (1×10^6) were plated on 10 cm plastic tissue culture plates precoated with 0.01% poly-L-lysine and 1 μg/ml fibronectin. The medium was changed every 3–4 d. Cells were passaged at 50–75% confluence by incubating them briefly in warm PBS and scraping them with a cell scraper.

Immunocytochemistry. For immunofluorescence double-staining the cells were cultured on Lab-Tek slides (Nunc, Naperville, IL) that had been precoated with 0.01% poly-L-lysine and 1 μg/ml fibronectin. The cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.5% Triton X-100 in PBS for 5 min; then they were blocked in 5% bovine serum albumin in Tris-buffered saline (TBS) for 10 min and incubated with primary antibodies at 37°C overnight, followed by Oregon Green-conjugated anti-mouse antibody and Texas Red-conjugated anti-rabbit antibody.

For dephosphorylation, fixed cells were treated after the blocking step with 256 U/ml alkaline phosphatase (Sigma) at 37°C overnight. After incubation the cells were washed with warm TBS, reblocked with 5% bovine serum albumin in TBS for 5 min, and incubated with primary and secondary antibodies as above.

Preparation of cell samples and rat brain homogenate. Cells were cultured up to $\sim$70–90% confluence in 10-cm-diameter dishes. For harvesting, the culture medium first was changed to cold (4°C) PBS, and the cells were collected immediately with a cell scraper and centrifuged at 800 × *g* at 4°C for 5 min. In most cases the resulting cell pellets were lysed in 0.4% SDS and 0.4% β-mercaptoethanol (BME) solution, immediately probe-sonicated, and boiled for 5 min. The lysates were aliquoted and stored at −85°C until use. For detection of tau isoforms and GSK-3 assay, the cells were lysed on ice for 30 min in lysis buffer [50 mM Tris, pH 8.2, for tau isoform analysis and pH 7.4 for GSK-3 assay; 0.1% Triton X-100; 20 mM NaCl; 1 mM phenylmethane-sulfonylfluoride (PMSF); and (in μg/ml) 5 leupeptin, 5 aprotinin, 2 pepstatin A, and 50 phosphoramidon], and centrifuged at 200,000 × *g* for 30 min. The resulting extracts were aliquoted and stored as above. The protein concentrations were determined by the modified Lowry method of Bensadoun and Weinstein (1976).

For the detection of tau isoforms, cell extracts (15 μg) from adult and fetal progenitor cells and from the cortex of a 3-month-old rat were mixed with alkaline phosphatase in the proportion of 2 μg of sample per 1 U of alkaline phosphatase, adjusted to 156–2000 U/ml with lysis buffer, and incubated at 37°C overnight. The incubation was stopped by adding the appropriate amount of SDS–BME solution and immediate boiling it for 5 min.

Treatment with growth factors. Cells that had been cultured with 10 ng/ml FGF-2 in 10-cm-diameter dishes were used to study the effects of FGF-2. On the starting day (experimental day 0), the medium was changed to new Neurobasal A/B27 (or Neurobasal/B27 for fetal stem cells) containing various concentrations of FGF-2. The medium was changed on experimental days 2, 4, and 6 with the same medium, and the cells were lysed on experimental day 7.

For transformation to astrocytes the cells were cultured with 10 ng/ml FGF-2 on Lab-Tek slides. On experimental day 0 the medium was changed to new Neurobasal A/B27 containing both 10 ng/ml ciliary neurotrophic factor (CNTF; Sigma) and FGF-2. The next day (day 1) the cells received fresh medium containing either 10 ng/ml CNTF alone or CNTF with 20 ng/ml FGF-2. Further medium changes were done on experimental days 3, 5, and 7, and the cells were fixed on experimental day 8 and double-stained as described above.

Radio immunoblots. Indicated amounts of protein samples were electrophoresed on at least triplicate SDS-polyacrylamide gels (8 × 6 × 0.75 cm; 6, 10, or 5–10% acrylamide), transferred to Immobilon membranes (Millipore, Bedford, MA), and probed with primary antibodies. To assay the degree of phosphorylation at the Tau-1 site, we treated the membranes with or without alkaline phosphatase (196 U/ml) at 37°C for 8 hr in dephosphorylation buffer [containing (in mM) 50 Tris, pH 8.2, 2 MgCl$_2$, and 1 PMSF plus (in μg/ml) 5 leupeptin, 5 aprotinin, 2 pepstatin A, and 50 phosphoramidon] before the application of Tau-1. Bound antibodies were probed with ^{125}I-conjugated anti-mouse or anti-rabbit IgG. The radio immunoblots were scanned with a Fuji BAS 1500 Bio Image analyzer (Raytest USA, Wilmington, DE). Images were processed with the Tina software, and the strength of immunostaining was expressed as pixels per square length (PSL). Tau levels (micrograms of
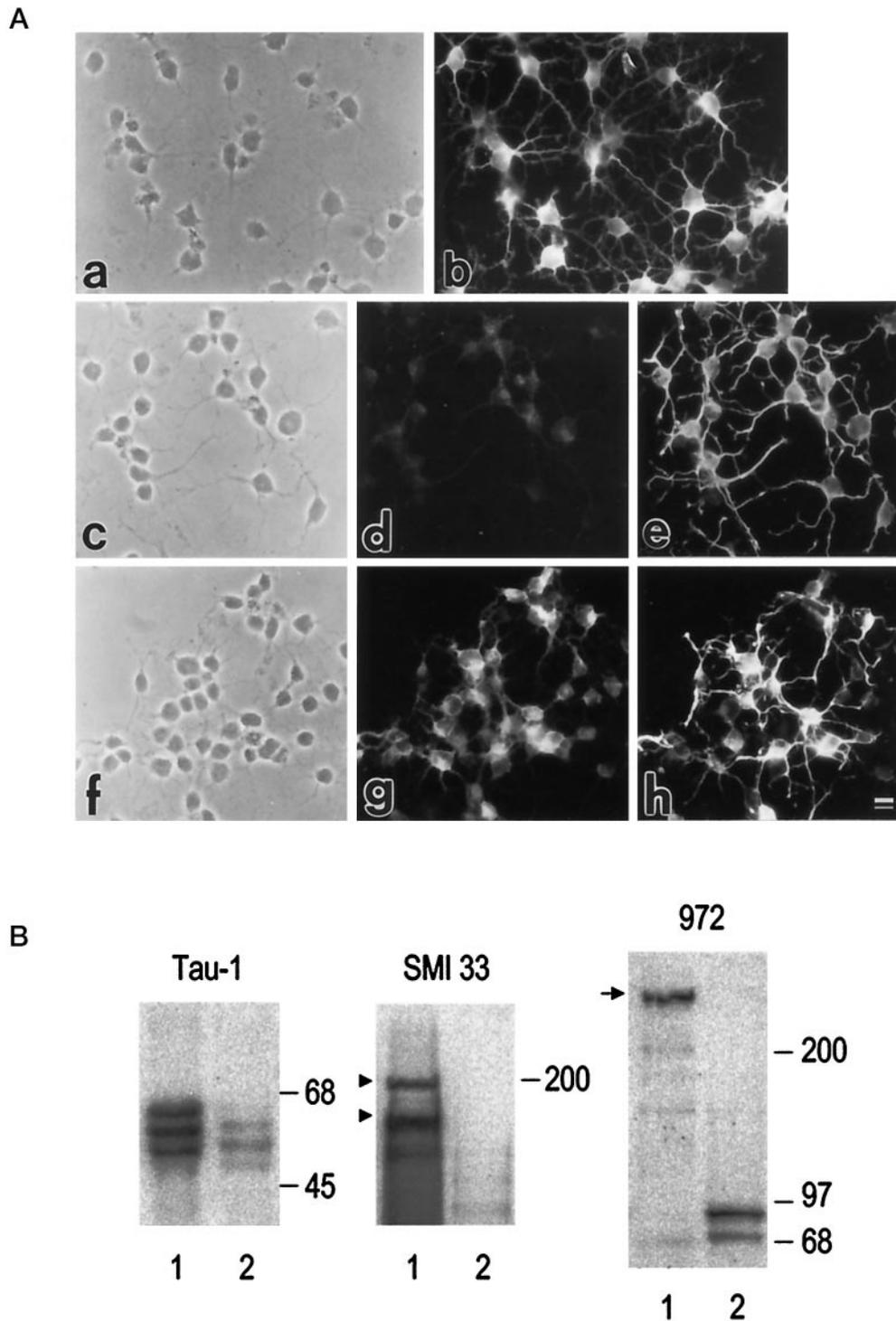
Figure 1. Proliferating adult hippocampal progenitor cells express not only nestin and MAP-2C but also tau, with the Tau-1 site phosphorylated. *A*, Expression of tau and nestin in proliferating adult hippocampal progenitor cells. Adult hippocampal progenitor cells (passage 7) were cultured with 10 ng/ml FGF-2 on Lab-Tek slides and stained as described in Materials and Methods. *a*, Phase contrast; *b*, anti-tau serum R134d. *c–h*, Cells were double-stained with Tau-1 and nestin (#133) antibodies without (*c–e*) and with (*f–h*) alkaline phosphatase pretreatment; (*c, f*) phase contrast; (*d, g*) Tau-1; (*e, h*) nestin (#133). Note that most adult progenitor cells were tau-positive. After alkaline phosphatase treatment most of the nestin-positive cells also became Tau-1-positive, indicating that tau in adult progenitor cells is phosphorylated at the Tau-1 site. Scale bar, 10 μm. *B*, Western blot analysis of lysates from adult progenitor cells and rat brain. Lysates (15 μg) from a 3-month-old rat brain tissue (*1*) and adult hippocampal progenitor cells (*2*) were applied on 5–10% SDS-polyacrylamide gels. Transferred membranes were pretreated with alkaline phosphatase and analyzed with antibodies Tau-1 (anti-dephosphorylated tau), SMI 33 (anti-dephosphorylated neurofilament H and M), and 972 (anti-dephosphorylated MAP-2A–C). Note that adult progenitor cells express tau and MAP-2C, but not the high-molecular-weight MAP-2 (*arrow*) and neurofilament H and M (*arrowheads*).
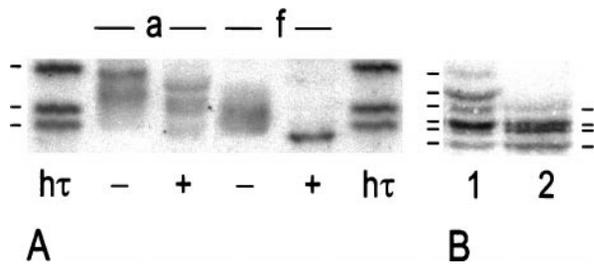
Figure 2. Adult hippocampal progenitor cells express adult isoforms of tau, and fetal cells express only the fetal tau isoform. *A*, Tau isoforms expressed by adult and fetal hippocampal progenitor cells. Extracts (15 μg) from adult (—*a*—) and fetal (—*f*—) progenitor cells were incubated *in vitro* with (+) or without (−) alkaline phosphatase and analyzed by Western blots with phospho-independent polyclonal antibody 92e. *h*τ, Recombinant human tau 39, 24, and 23 (10 ng each). *Bars* indicate their apparent molecular weights (from *top* to *bottom*) of 62, 52, and 48 kDa, respectively. Note that, after treatment with alkaline phosphatase *in vitro*, tau from fetal cells was resolved into a single band, but tau from adult cells still showed several major bands. *B*, Comparison of the tau isoform profiles of adult hippocampal progenitor cells and rat brain tissue. Extracts (15 μg) from a 3-month-old rat (*1*) and adult hippocampal progenitor cells (*2*) were incubated *in vitro* with alkaline phosphatase, applied on a 6% gel, and analyzed with the Tau-1 antibody. *Bars left* and *right* indicate the apparent molecular weights of tau isoforms (from *top* to *bottom*) of 61, 58 (only on *left*), 53, 50, 46, and 43 kDa, respectively. The 58 kDa tau isoform in the progenitor cells could be observed only if the membrane was overexposed (data not shown).

tau/mg total protein) were determined by comparing the PSLs of known amounts of recombinant tau and of the taus in total cell lysates in the blots treated with alkaline phosphatase and stained with Tau-1. Phosphorylation at the Tau-1 epitope (percentage of phosphorylation) was calculated by the following formula: [PSL of Tau-1 in alkaline phosphatase-treated blot (total tau) − PSL of Tau-1 in the untreated blot (tau not phosphorylated at the Tau-1 site)]/PSL of Tau-1 in alkaline phosphatase-treated blot (total tau) × 100.

GSK-3 assay. Activities of GSK-3 were determined as described previously (Pei et al., 1997). Aliquots of 50 μg of cell extract protein were diluted to 250 μl with lysis buffer and mixed with 250 μl of TBS, 300 mM NaCl, and 1 mM PMSF with or without 2 μl of anti-GSK-3 serum R133. After incubation at 4°C for 8 hr, 20 μl of immobilized G-protein (Pierce, Rockford, IL) was added to the mixtures, which were incubated further at 4°C for 2 hr and then centrifuged. The precipitates were washed three times and suspended in 50 μl of 50 mM Tris, pH 7.4, containing 10 mM MgCl$_2$ and 1 mM PMSF. Equal amounts (10 μl) of precipitate suspension, 800 μM [γ-^{32}P] ATP, 0.4 mg/ml phosphoglycogen synthase peptide 2 (UBI, Lake Placid, NY), and 120 mM Tris, pH 7.4, containing (in mM) 40 MgCl$_2$, 40 NaF, 4 Na$_3$VO$_4$, 8 EGTA, and 40 BME were mixed and incubated at 30°C for 30 min. All assays were performed in triplicate. Reactions were stopped with 40 μl of 300 mM *o*-phosphoric acid, and the mixtures were applied to phosphocellulose units (Pierce). The filters were washed three times with 75 mM phosphoric acid, dried, and counted. For negative control the precipitates that had been incubated without antibody were used. GSK-3 activity was obtained by subtracting the counts in the absence of antibody from the counts in the presence of antibody.

RESULTS

Adult hippocampal progenitor cell cultures express nestin, MAP-2C, and phosphorylated tau

Hippocampal cells were isolated from adult brain and cultured with 5 ng/ml FGF-2 for the first 3 months. In the first few weeks the cultures were heterogeneous, containing one major type of cells with small round cell bodies and multiple branched, thin processes. A change of FGF-2 from 5 to 10 ng/ml dramatically increased the speed of proliferation of these cells.

After the fifth passage, almost all of the cells immunocytochemically stained intensely with polyclonal antibody 134d for

tau, which recognizes tau in a phosphorylation-independent manner (Fig. 1*Aa,Ab*). In contrast, these cells were stained only poorly with antibody Tau-1, which recognizes only tau that is not phosphorylated at the Tau-1 epitope (Fig. 1*Ac,Ad*). However, when the fixed cells were dephosphorylated with alkaline phosphatase before the application of Tau-1 antibody, the staining pattern changed dramatically (Fig. 1*Af,Ag*) in that almost all of the cells were immunostained intensely. Both Tau-1 and 134d antibodies stained cell bodies and processes, but not the nuclei, suggesting that tau in these cells is mostly cytosolic. Most cells also were stained with antibody 972 to dephosphorylated MAP-2A–C (data not shown), whereas only a rare cell was stained with GFAP antibodies (data not shown). Almost all of the cells also were stained with monoclonal (data not shown) and polyclonal antibodies to nestin (Fig. 1*Ae,Ah*), a marker for immature neural cells. In some instances the nestin staining increased after dephosphorylation (Fig. 1*Ah*). Presently, it is not known whether this treatment nonspecifically increased the antibody accessibility to nestin by unmasking it or whether nestin antibody #130 is somewhat phospho-dependent.

Western blot analysis of the cell lysates with monoclonal antibody Tau-1 after dephosphorylation of the blot revealed several immunopositive bands in the 48–62 kDa area but with slightly different mobilities from the major tau bands in rat brain (Fig. 1*B*, *Tau-1*). In contrast to rat brain homogenate, which also contained the high-molecular-weight isoforms of MAP-2 and the H and M neurofilament subunits, in the cell lysates only MAP-2C (~70 kDa) and a band of ~90 kDa, most probably a MAP-2C-derivative variant (Kalcheva et al., 1997), but not MAP-2A and MAP-2B, were detected with antibody 972 (Fig. 1*B*, *972*). No neurofilament polypeptides were labeled with antibody SMI 33 in the cell lysates (Fig. 1*B*, *SMI 33*). These data demonstrate clearly that the cells we cultured were morphologically and immunocytochemically indistinguishable from adult hippocampal progenitor cells or the stem cells described previously (Gage et al., 1995; Johe et al., 1996; Palmer et al., 1997); these cells will be called "progenitor cells" below.

Expression of tau isoforms in adult and fetal hippocampal progenitor cells

To study the isoform composition of tau in adult and fetal progenitor cells, we compared polypeptide patterns of tau in cell extracts by Western blots developed with phospho-independent polyclonal antibody 92e. Cell extracts were incubated *in vitro* with or without alkaline phosphatase before application on the gel (Fig. 2*A*). Without dephosphorylation, tau in the cell extracts from both adult and fetal progenitor cells showed several immunoreactive bands ranging from 48 to 62 kDa. However, on *in vitro* dephosphorylation, tau from adult progenitor cells was resolved into three major distinct bands. In contrast, tau from fetal progenitor cells showed only a single band of ~43 kDa, suggesting that adult hippocampal progenitor cells express adult isoforms of tau, whereas fetal hippocampal progenitor cells express only the fetal tau isoform (Fig. 2*A*). On Western blots that used 6% gradient gels and Tau-1 antibody, tau from the adult progenitor cells was resolved into five isoforms but in different ratios from those seen in rat brain (Fig. 2*B*). Tau from adult rat brain was resolved into six bands of 61, 58, 53, 50, 46, and 43 kDa. In contrast, tau from adult progenitor cells resolved into four major polypeptides of 53, 50, 46, and 43 kDa and a weakly labeled polypeptide of 58 kDa, which was visible only when the ^{125}I blot was overexposed. An identical Tau-1-immunoreactive band pat-
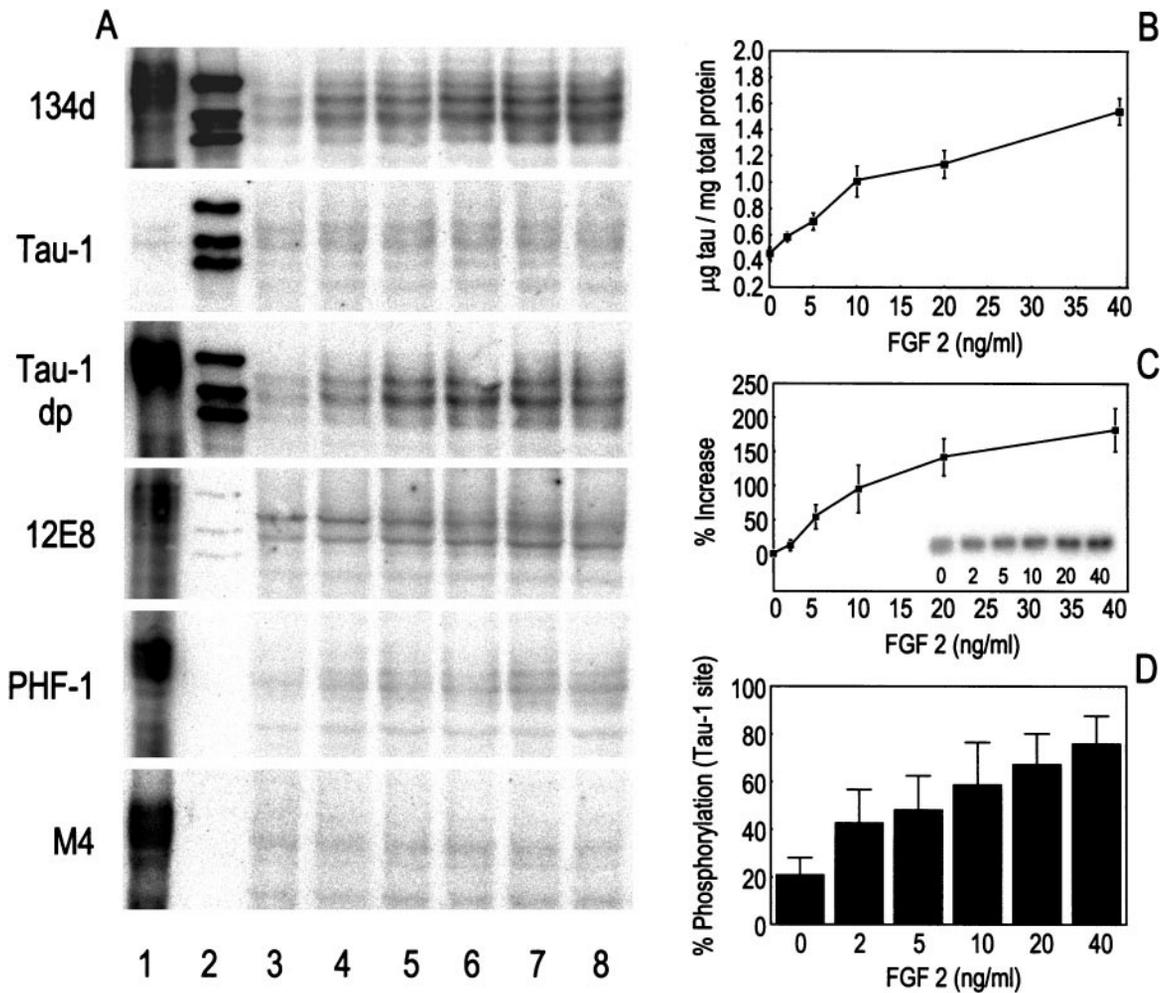
Figure 3. FGF-2 increases the tau and tubulin expressions and the degree of tau phosphorylation at the Tau-1 site in adult hippocampal progenitor cells. *A*, Western blot analysis of cell lysates from adult hippocampal progenitor cells cultured with increasing concentrations of FGF-2. Adult progenitor cells (passage 8) cultured with 0, 2, 5, 10, 20, and 40 ng/ml of FGF-2 for 7 d were lysed and analyzed with phosphorylation-independent antibody 134d and with phosphorylation-dependent antibodies Tau-1 without (*Tau-1*) or with (*Tau-1 dp*) alkaline phosphatase treatment on the membranes, 12E8, PHF-1, and M4. *Lane 1*, AD P-tau, 3 μg; *lane 2*, mixture of recombinant human tau 23, 24, and 39, 10 ng each. *Lanes 3–8*, Cell lysates, 12.5 μg, from the cultures with different concentrations of FGF-2: *lane 3*, 0 ng/ml; *lane 4*, 2 ng/ml; *lane 5*, 5 ng/ml; *lane 6*, 10 ng/ml; *lane 7*, 20 ng/ml; *lane 8*, 40 ng/ml. *B*, Tau levels increase, depending on the FGF-2 concentrations. Tau levels in adult hippocampal progenitor cells (passage 8) were determined from the radio immunoblots as described in Materials and Methods ($n = 3$). Absolute tau amounts (micrograms of tau per milligram total protein) were derived by using recombinant human tau as a standard. Similar results also were obtained with adult progenitor cells that had been passaged five times. *C*, Tubulin levels increase with increased FGF-2 concentration. Cell lysates (6 μg) were analyzed by Western blots with tubulin monoclonal antibody DM1A (*inset*). The percentage of increase in immunoreactivity was calculated on the basis of the value of FGF-2 at 0 ng/ml ($n = 3$), which was taken as 0%. *D*, The phosphorylation of tau at the Tau-1 site increases with increased FGF-2 concentration. The degree of phosphorylation at the Tau-1 site was determined from the Western blots as described in Materials and Methods ($n = 3$). Note that FGF-2 had a strong effect on the phosphorylation of tau at the Tau-1 site in adult progenitor cells.

tern was observed even after incubation with several-folds of alkaline phosphatase (1000–2000 U/ml; data not shown). These data show that undifferentiated proliferating adult hippocampal progenitor cells do express adult isoforms of tau, but the ratio at which the isoforms are expressed is different from that of rat cerebrum.

Regulation of expression and phosphorylation of tau by FGF-2

Adult progenitor cells

To determine the effect of FGF-2 on tau expression, we incubated cells with increasing concentrations of FGF-2, ranging from 0 to 40 ng/ml for 7 d, and we determined the levels of tau in the cell lysates by [^{125}I] Western blots as described in Materials and Methods. Using phospho-independent tau antibody 134d, we found that

FGF-2 markedly increased, in a concentration-dependent manner, the level of total tau expression (Fig. 3*A,B*). This effect also was observed in Tau-1 blots treated with alkaline phosphatase. In cultures without FGF-2, the tau level was ~0.45 μg/mg of total protein. This amount increased with an increase in FGF-2 concentration and reached ~1.5 μg/mg at 40 ng/ml of FGF-2 concentration (Fig. 3*B*). Furthermore, in addition to the FGF-2-dependent increase of total tau, tubulin levels also increased (Fig. 3*C*).

As shown in Figure 1 by immunocytochemistry, tau in adult progenitor cells is phosphorylated at the Tau-1 site. This also was confirmed by immunoblot analysis (Fig. 3*A*). Radio immunoblot assays demonstrated that the degree of phosphorylation at the Tau-1 site also is regulated strongly by the concentration of FGF-2 (Fig. 3*D*). In FGF-2-depleted cultures the phosphorylation
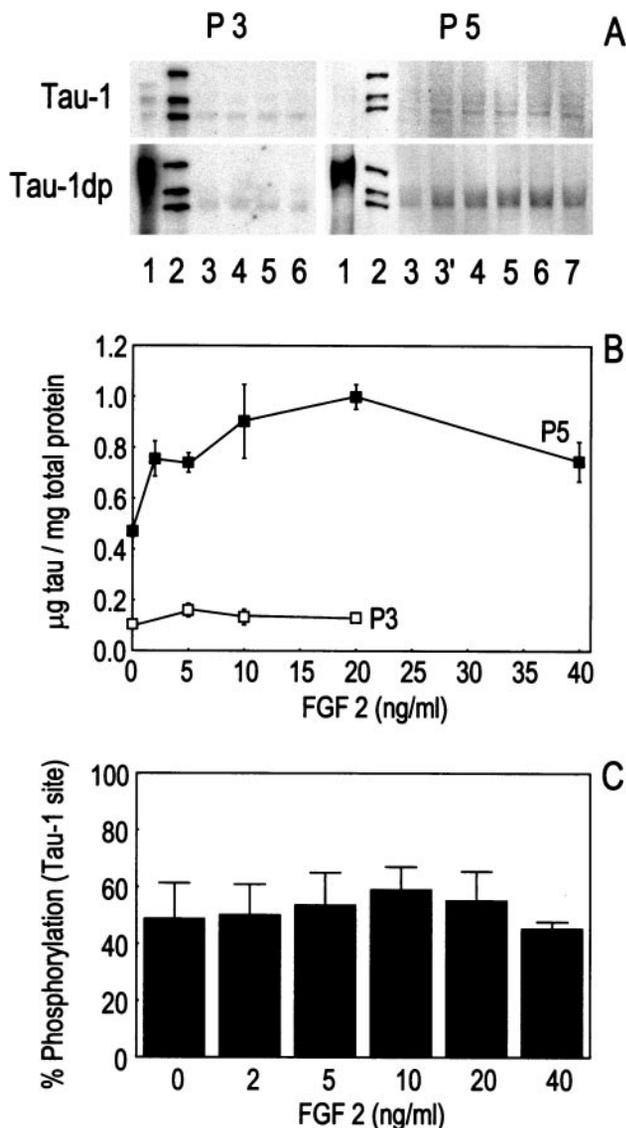
Figure 4. In fetal hippocampal progenitor cells, FGF-2 increases the expression of tau only in cultures of higher passages but has no effect on the phosphorylation at the Tau-1 site. *A,* Western blot analysis of cell lysates from fetal hippocampal progenitor cells. Fetal hippocampal progenitor cells, passage 3 (*P3*) and passage 5 (*P5*), cultured with increasing concentrations of FGF-2 for 7 d were lysed and analyzed with Tau-1 without (*Tau-1*) or with (*Tau-1 dp*) alkaline phosphatase treatment on the membranes. *Lane 1,* AD P-tau, 3 µg; *lane 2,* mixture of recombinant human tau 23, 24, and 39, 10 ng each. *Lanes 3–7,* Cell lysates (12.5 µg) from the cultures with increasing concentrations of FGF-2: *lane 3,* 0 ng/ml; *lane 3′,* 2 ng/ml; *lane 4,* 5 ng/ml; *lane 5,* 10 ng/ml; *lane 6,* 20 ng/ml; *lane 7,* 40 ng/ml. Note that fetal progenitor cells in P3 cultures expressed fetal tau very weakly, and FGF-2 had no effect on its expression or phosphorylation. In contrast, the cells in P5 cultures expressed tau abundantly, and FGF-2 increased its expression. *B,* Tau levels in fetal hippocampal progenitor cells increase with increased concentration of FGF-2 only in later passages. Tau levels were analyzed from the Western blots and expressed by using the same methods as for Figure 3*B.* Tau levels increased 5- to 10-fold in P5 cultures as compared with P3 cultures. Moreover, FGF-2 altered tau expression only in P5 culture. *C,* Degree of phosphorylation at the Tau-1 site in fetal hippocampal progenitor cells is independent of the concentration of FGF-2. The degree of phosphorylation in the P5 cultures was determined as described in Materials and Methods. Similar results also were obtained in P3 cultures (data not shown). Note that FGF-2 had no effect on tau phosphorylation at the Tau-1 site.

at the Tau-1 site was only ~20% but increased to close to 80% at an FGF-2 concentration of 40 ng/ml. A small increase in phosphorylation also was seen at the 12E8 and PHF-1 sites concurrent with increased FGF-2 (Fig. 3*A*). However, this increase in phosphorylation was not observed when normalized against total tau (as determined with Tau-1 after dephosphorylation), suggesting that the increased tau is in the form of the protein phosphorylated at the 12E8 and PHF-1 sites. Antibody M4 recognized tau weakly with the immunostaining, even slightly decreasing with increasing tau concentrations. Thus it is indicated that this site is not phosphorylated significantly in adult hippocampal progenitor cells as a response to FGF-2 (Fig. 3*A*).

Fetal progenitor cells

The effect of FGF-2 on the expression and the phosphorylation of tau also was investigated in progenitor cells from fetal rat hippocampus. During the first three passages the total tau appeared to decrease in the fetal progenitor cells (data not shown), as described previously (Sah et al., 1997). The tau level in the cells from the third passage was ~0.1 µg/mg of total protein, and FGF-2 had no effect on the total tau expression or phosphorylation at the Tau-1 site (Fig. 4*A,B*). However, in the fifth passage even tau from cells that had been grown for 1 week in FGF-2-depleted medium was increased fivefold to approximately the same level as the similarly treated adult progenitor cells (compare Figs. 3*B*, 4*B*). The addition of FGF-2 to these cultures resulted in an increase in the tau level, but this increase was only one-half of that seen in the adult cells, even at 20 ng FGF-2/ml, the maximal point of increase. At 40 ng/ml of FGF-2 the tau content decreased. The reason for this negative switch is not understood at present. A possibility is that the inhibition of tau expression beyond a certain maximal point represents a regulatory mechanism by the fetal cells. In contrast to adult cells, 50% of tau already was phosphorylated at the Tau-1 site in the FGF-2-depleted fetal cells, and the addition of FGF-2 did not have any effect on Tau-1 phosphorylation (Fig. 4*C*). Thus, the regulation of expression and phosphorylation of tau by FGF-2 between adult and fetal hippocampal progenitor cells was different.

Even cultures grown for up to 3 months with multiple passages expressed mostly fetal tau, suggesting that factors other than FGF-2 are required to induce adult isoforms of tau in fetal hippocampal progenitor cells.

Differentiated astrocytes

To elucidate whether the effect of FGF-2 on tau is limited to undifferentiated progenitor cells or also might extend to cells differentiated into astrocytes, we stimulated progenitor cells with 10 ng/ml of CNTF with or without the addition of FGF-2 (20 ng/ml), as described in Materials and Methods. CNTF converts adult neural progenitor cells to astrocytes (Johe et al., 1996). After stimulation for 8 d the cells were double-stained with monoclonal antibody to GFAP and polyclonal tau antibody 134d. In cultures stimulated with only CNTF on alternate days, ~30–40% of the cells were GFAP-positive. The GFAP-positive cells contained no or only very low tau immunoreactivity (Fig. 5*b,c*). In cultures that were stimulated simultaneously with CNTF and FGF-2, the same proportion of cells was stained with GFAP. However, most of the GFAP-positive astrocytes also were strongly tau-positive, at approximately the same intensity as in undifferentiated progenitor cells (Fig. 5*e,f*). Immunocytochemistry with Tau-1 antibody with or without alkaline phosphatase treatment showed that, as in the undifferentiated adult progenitor
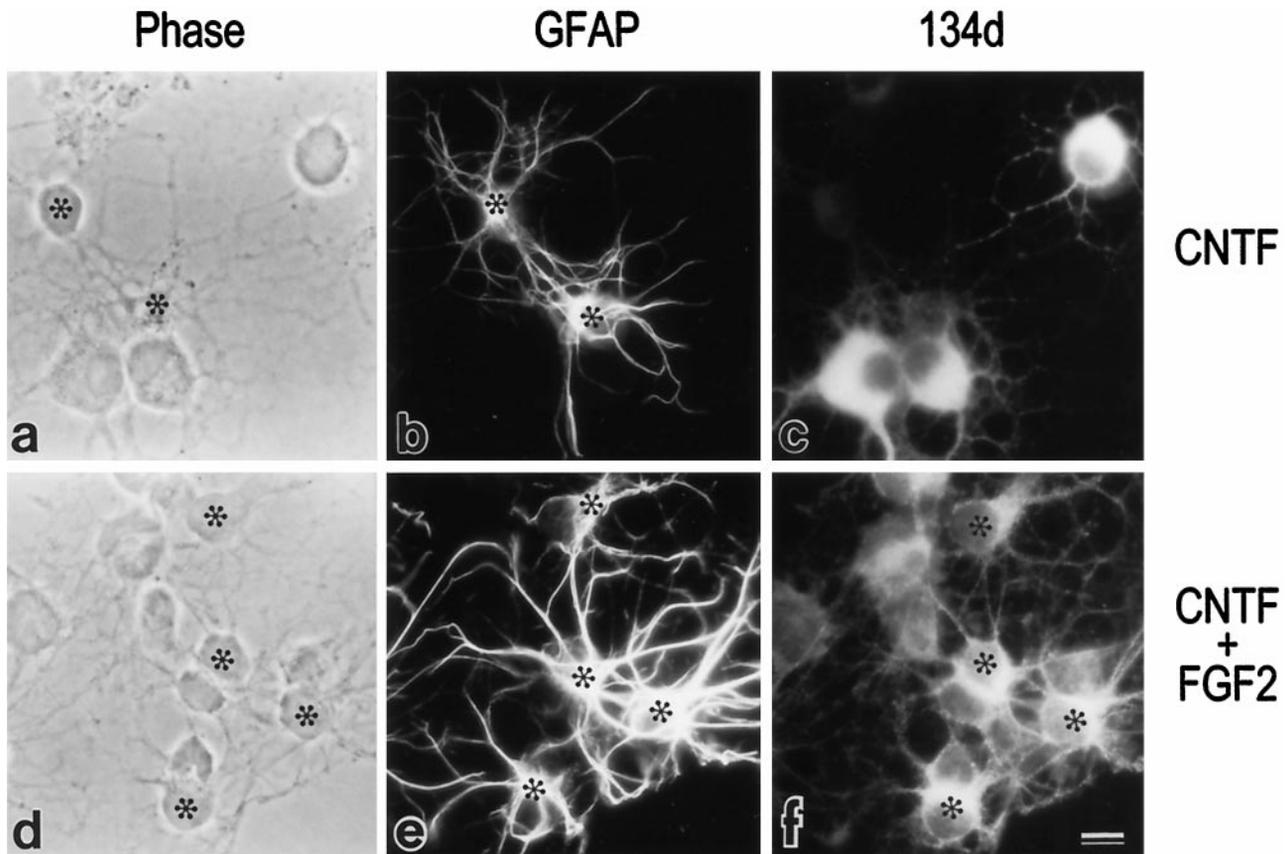
Figure 5.   Effect of FGF-2 on the expression of tau in differentiated astrocytes. Adult hippocampal progenitor cells cultured with 10 ng/ml CNTF alone (*a–c*) or, in addition, with 20 ng/ml FGF-2 (*d–f*) for 8 d as in Materials and Methods were double-stained with mono-GFAP and 134d. *a, d*, Phase contrast; *b, e*, mono-GFAP; *c, f*, 134d. In the cultures stimulated with CNTF alone, differentiated astrocytes were tau-negative (*c*) or very weakly tau-positive (data not shown). In contrast, in cultures stimulated with both CNTF and FGF-2, most astrocytes expressed tau strongly with the same intensity as undifferentiated progenitor cells. *Asterisks* show differentiated astrocytes. The tau-positive cells in *c* and *f* most probably represent undifferentiated progenitor cells (GFAP-negative). Scale bar, 10 μm.

cells, the Tau-1 site was hyperphosphorylated (data not shown). These findings indicate that FGF-2 affects tau expression and phosphorylation even in adult differentiated astrocytes.

Mechanism of the phosphorylation of tau by FGF-2 in adult progenitor cells

To date, MAPK, GSK-3, and Cdk-5 are the three major candidate kinases that have been reported to induce tau phosphorylation at the Tau-1 site *in vitro* (Drewes et al., 1992; Hanger et al., 1992; Vulliet et al., 1992; Singh et al., 1995b). Therefore, to identify the protein kinase(s) that might be involved in the FGF-2-induced tau hyperphosphorylation in adult progenitor cells, we examined the effect of inhibitors specific to the above kinases. After the indicated periods of incubation with each kinase inhibitor and 10 ng/ml FGF-2, the cells were lysed and analyzed with Tau-1 antibody on Western blots that had been treated or untreated with alkaline phosphatase (Fig. 6*A*). PD 98059, which inhibits the activation of MAPK/MAPK kinase (Alessi et al., 1995), and butyrolactone I, which inhibits the activities of cdks (Hosoi et al., 1995), failed to inhibit the phosphorylation of tau induced by FGF-2 (Fig. 6*A*). However, lithium, which inhibits GSK-3 activity (Klein and Melton, 1996), inhibited the phosphorylation of tau and resulted in a mobility shift of tau bands (see Fig. 6*A*; compare *lane 2* with *lanes 1, 3, 4*). For further confirmation the cells were treated at different concentrations of LiCl for 6 hr, and the percentage of phosphorylation was determined as de-

scribed in Materials and Methods (Fig. 6*B*). The degree of phosphorylation at the Tau-1 site was decreased by increasing concentrations of LiCl in a dose-dependent manner, suggesting that GSK-3 is the kinase involved in the FGF-2 signaling pathways.

To confirm the involvement of GSK-3 in the FGF-2-mediated hyperphosphorylation of tau, we assayed the GSK-3 activity, using phosphoglycogen synthase peptide 2 as the specific substrate. GSK-3 was immunoprecipitated from the cell extracts, and its kinase activity was assayed. As expected, FGF-2 increased GSK-3 activity in a dose-dependent manner (Fig. 6*C*), again supporting the concept that FGF-2 induces tau phosphorylation by activating GSK-3.

DISCUSSION

The nature of the extracellular signal that regulates the expression and phosphorylation of tau is not understood. The present study shows for the first time that (1) neural proliferating progenitor cells from adult rat brain stably express adult isoforms of tau, (2) that the expression and the phosphorylation at the Tau-1 site of the adult tau isoforms are regulated by FGF-2, (3) that the kinase responsible for the FGF-2-mediated tau phosphorylation is GSK-3, and (4) that FGF-2 induces expression and phosphorylation of tau in differentiated astrocytes. In contrast, fetal progenitor cells express mostly fetal tau, and FGF-2 regulates only its
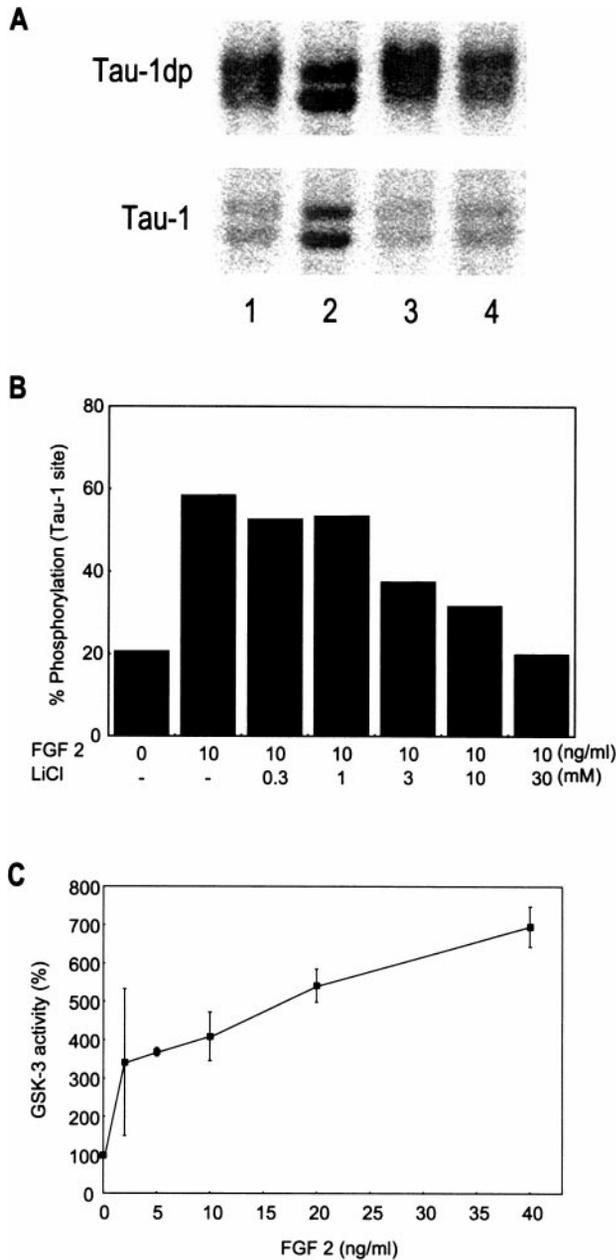
Figure 6. Phosphorylation of tau induced by FGF-2 is mediated by GSK-3. *A*, Effect of kinase inhibitors on the phosphorylation of tau induced by FGF-2. Adult progenitor cells treated with butyrolactone I (30 μg, 6 hr), PD98059 (50 μg, 60 min), or LiCl (30 mM, 3 hr) and 10 ng/ml FGF-2 were lysed and analyzed on Western blots that had been treated with (*Tau-1 dp*) or without (*Tau-1*) alkaline phosphatase. *Lane 1*, Untreated; *lane 2*, LiCl; *lane 3*, butyrolactone I; *lane 4*, PD98059. Note that the phosphorylation at the Tau-1 site induced by FGF-2 was inhibited only by lithium, an inhibitor of GSK-3 (no upward mobility shift in *lane 2* in *top panel* as compared with *lanes 1, 3, 4*). *B*, Dose-dependent inhibitory effect of lithium on FGF-2-mediated tau phosphorylation. Adult progenitor cells treated with indicated concentrations of LiCl for 6 hr in 10 ng/ml FGF-2 were lysed, and the percentage of phosphorylation was determined from Western blots as described in Materials and Methods. The data points without LiCl are from Figure 3*D*. The percentage of phosphorylation was decreased by lithium in a dose-dependent manner. *C*, FGF-2 increases GSK-3 activity. GSK-3 activities were measured by using extracts from the cells that had been cultured in the indicated concentrations of FGF-2 for 7 d. The GSK-3 activity (%) was calculated on the basis of the value of FGF-2 at 0 ng/ml (*n* = 3). FGF-2 increases GSK-3 activity in a dose-dependent manner.

expression, but not its phosphorylation. The production of adult tau isoforms together with the ability to differentiate, on stimulation, into neuronal and/or glial cells indicates the potential of adult progenitor cells as a cell culture model to study adult neurodegenerative diseases that are characterized by an accumulation of hyperphosphorylated tau.

No tau immunoreactivity has been reported previously in undifferentiated adult progenitor cells. In this study, however, we found that these cells express abundantly the adult isoforms of tau, with the Tau-1 site highly phosphorylated. This phosphorylation was the very reason that the presence of tau in these cells had been overlooked previously. Most of the previous studies had used Tau-1 antibody, which recognizes tau only when it is dephosphorylated at the Tau-1 site. Indeed, the same results were obtained in the present study when Tau-1 was used without any previous treatment with alkaline phosphatase (see Fig. 1*A*).

The present study gives rise to several issues. First, tau expression not only is restricted to differentiated neurons but also occurs in actively dividing progenitor cells and astrocytes derived from them. Second, the expression of adult tau isoforms does not necessarily indicate the maturity of a cell. This point is demonstrated in the progenitor cells from adult hippocampus, which, although immature (nestin-positive), still produce most of the adult tau isoforms but in different proportions from those in adult rat brain tissue. On the other side of the spectrum are the progenitor cells from fetal brain, which do not seem to switch to adult tau. In contrast, in primary cultures of neonatal rat brain, changes in tau mRNA splicing and production of adult tau isoforms have been observed during 6–12 d in culture (Pizzi et al., 1995). Most likely, fetal progenitor cells are too immature developmentally and lack the factors to make *in vitro* the switch from fetal to adult tau.

The marked effect of FGF-2 on the expression of tau in progenitor cells from adult rat brain indicates the role of FGF-2 as a regulator of tau. This effect of FGF-2 on tau might be acquired during later stages of the development, because the increase in tau expression in the progenitor cells from fetal brain could not be observed until the fifth passage. The dynamic effect of FGF-2 on tau expression also may be of importance for postmitotic neurons. FGF-2 has been reported to have an effect on axonal formation (Walicke et al., 1986), especially axonal branching (Miyagawa et al., 1993) and *in vivo* sprouting in cholinergic neurons (Fagan et al., 1997). Tau is one of the major MAPs in mature neurons and is expressed abundantly in axons (Binder et al., 1985). Therefore, FGF-2 possibly may have an effect on the regulation of axonal formation, generation, and, especially, regeneration after injury by affecting tau expression and phosphorylation in adult brain.

It has been shown previously that progenitor cells differentiate into astrocytes by stimulation with CNTF, both in the presence or absence of FGF-2 (Johe et al., 1996). We studied the effect of FGF-2 on tau in astrocytes derived from CNTF-treated adult progenitor cells. As shown in Figure 5, tau is present in cultured astrocytes when they are treated with FGF-2. The tau proteins in these differentiated astrocytes also are regulated by FGF-2 as well as those in undifferentiated progenitor cells, suggesting that the effect of FGF-2 on the expression of adult tau may be more general in adult CNS. Although the mechanisms are still unclear, in some pathological conditions, including progressive supranuclear palsy, corticobasal degeneration, Pick's disease, dementia pugilistica, and Alzheimer Disease, abnormally phosphorylated tau-positive astrocytes or astrocytic processes have been reported

to appear in the affected areas of the brain (for review, see Chin and Goldman, 1996), sometimes in parallel with overexpression of FGF-2 (Stopa et al., 1990).

In the present study we found that FGF-2 upregulates the phosphorylation of tau at the Tau-1 site in progenitor cells from adult hippocampus. This increase in tau phosphorylation is attributable mainly to the increased levels of phosphorylated tau (Fig. 3*A*, compare *Tau-1* with *Tau-1 dp*). Interestingly, this pattern of increase in phosphorylated tau is similar to that in AD brain (see Khatoon et al., 1992). Furthermore, the present study shows that the increase in tau phosphorylation at this site corresponded to an increase in the activity of GSK-3 but neither MAPK nor cdks, suggesting that GSK-3 activity might be regulated by the FGF-2 signaling pathway. Unlike adult progenitor cells, these cells from fetal brain did not reveal any increase in tau phosphorylation in response to FGF-2 treatment, suggesting that the FGF-2 signaling pathway that regulates the GSK-3 activity is probably not developed in the fetal cells.

GSK-3 is believed to be a key kinase in AD involved in the hyperphosphorylation of tau, because it can induce Alzheimer-like phosphorylation in COS cells transfected with tau (Lovestone et al., 1994). *In vitro* Alzheimer-type phosphorylation of tau is induced by GSK-3 only in the presence of heparin or when tau is prephosphorylated with certain nonproline-dependent kinases (Moreno et al., 1995; Singh et al., 1995a,b; Sengupta et al., 1998). That not all characteristic AD tau epitopes are hyperphosphorylated in the progenitor cells with the conditions used in the present study may be attributable to differences in the kinase/phosphatase ratios or exogenous factors such as neurotrophins or lymphokines. However, in AD, with its decreased protein phosphatase activity (Gong et al., 1993, 1995), FGF-2 might be one of the factors that play an important role in the increased tau levels (Khatoon et al., 1992) and the formation and accumulation of highly phosphorylated tau in brain (Grundke-Iqbal et al., 1986a; Iqbal et al., 1989; Lee et al., 1991; Köpke et al., 1993). Indeed, in AD brain the FGF-2 level is elevated when compared with age-matched control brain (Stopa et al., 1990), and strong FGF-2 immunoreactivity has been observed in tau lesions such as neurofibrillary tangles and dystrophic and degenerating neurites of neuritic plaques (Cummings et al., 1993). Moreover, brain injury, one of the major risk factors of AD and probably the cause of dementia pugilistica, also is known to increase the expression of FGF-2 (Finklestein et al., 1988; Frautschy et al., 1991; Logan et al., 1992). Furthermore, continuous upregulation of FGF-2 in brain might be critical because it can induce not only an initial accumulation of hyperphosphorylated tau but also continuous activation of GSK-3. Increased GSK-3 activity has been reported to induce cholinergic dysfunction in primary cultured neurons (Hoshi et al., 1996) and apoptosis in PC12 cells (Pap and Cooper, 1998), both reported to occur in AD neuropathology. GSK-3 also is involved in the regulation of diverse transcription factors, which also might be affected by increased GSK-3 activity.

In conclusion, FGF-2 upregulates the expression and phosphorylation of tau in progenitor cells and differentiated astrocytes from adult, but not fetal, rat hippocampus. The hyperphosphorylation of tau produced by FGF-2 in these cells occurs via upregulation of GSK-3 activity but neither MAPK nor cdks activities. These findings identify a new role of FGF-2 both in normal neurobiology and in neurodegenerative diseases characterized by the accumulation of hyperphosphorylated tau.

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