The phosphorylation state of the microtubule-associated protein tau as affected by glutamate, colchicine and β -amyloid in primary rat cortical neuronal cultures

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The effects of the excitatory amino acid glutamate, the microtubule destabilizing agent colchicine, and β_{25-35} -amyloid peptide on the phosphorylation state of tau were studied in rat cortical neurons in primary culture. Using immunocytochemistry and Western-blot analysis, we demonstrated that a proportion of tau in these cultures is normally highly phosphorylated, but most of this tau fraction is dephosphorylated after treatment of the cultures with glutamate or colchicine, but not with β -amyloid; the glutamate- and colchicine-induced changes in tau phosphorylation commenced before cell death, as assessed by release of lactate dehydrogenase. Dephosphorylation of tau was readily revealed by using the monoclonal antibodies Tau.1 and AT8, which have phosphate-sensitive epitopes that both centre around serine-199 and -202 (numbering of the largest tau isoform). On Western blots and by immunocytochemistry, AT8 labelling strongly decreased after glutamate and colchicine treatments, whereas Tau.1 staining was more intense. Neurofilament monoclonal antibodies, including RT97, 8D8, SMI31 and

SMI310, all additionally known to recognize tau in a phosphorylation-dependent manner, also demonstrated that glutamate and colchicine treatments of the cultures induced a dephosphorylation of tau. We also showed immunocytochemically that there is an increase in tau immunoreactivity in neuronal perikarya in response to glutamate and colchicine treatment, and this occurs concomitantly with the dephosphorylation of tau. Treatment of the primary rat cortical neuronal cultures with β_{25-35} -amyloid peptide, under conditions which induce neuronal degeneration, did not induce a change in tau phosphorylation, and failed to act synergistically with glutamate to produce an increase in dephosphorylation of tau over that produced by glutamate treatment alone. These findings demonstrate that glutamate and colchicine induce tau dephosphorylation, as opposed to increased tau phosphorylation, which would be more indicative of Alzheimer-type neurodegeneration.

INTRODUCTION

The molecular events underlying neurodegeneration of Alzheimer's disease are not established, but it has been suggested that an excitotoxic mechanism mediated by excessive extracellular glutamate may be a contributory factor, and more recently the neurotoxicity of aggregated β -amyloid has received much attention (for review see [1]). In support of an excitotoxic mechanism, Mattson and colleagues have reported that glutamate and β -amyloid act synergistically to cause neuronal death *in vitro* and that this cell death is preceded by an increase in perikaryal tau immunoreactivity, which is also produced by treatment of neurons with colchicine [2,3]; these changes in perikaryal tau were suggested to mimic those observed in neurofibrillary tangle-bearing neurons in Alzheimer's disease [2]. Since at least many degenerating neurons in the Alzheimer's-disease brain exhibit a characteristic cytoskeletal pathology, particularly hyperphosphorylated paired helical filament (PHF) tau, we set out to investigate whether in cultured neurons neurotoxicity induced by glutamate, colchicine and β -amyloid produced changes in the phosphorylation state of tau that may parallel those occurring in Alzheimer's disease.

The microtubule-associated phosphoprotein tau is a major constituent of PHF, which constitute neurofibrillary tangles, a pathological hallmark of Alzheimer's disease [4,5]. PHF-tau differs from tau isolated from normal adult human brain in that it is resolved on SDS/PAGE as three to four bands of apparent molecular mass 60–68 kDa, instead of the six or more bands of apparent molecular masses between 55 and 62 kDa that are characteristic of normal adult brain tau [6–10]; PHF-tau can be induced to migrate like normal tau by treatment with alkaline phosphatase, suggesting that it is excessively phosphorylated [9–11]. PHF-tau has also been shown to be hyperphosphorylated through the use of a number of monoclonal antibodies that recognize specific phosphorylated epitopes and discriminate normal adult brain tau from PHF-tau [12,13]. By contrast, tau extracted from foetal brain comprises a single isoform that corresponds to the shortest adult brain tau species, and a fraction of this foetal tau is in an elevated state of phosphorylation, resembling the phosphorylation state of PHF-tau [14–16].

The majority of investigations of immunocytochemical changes in tau in response to excitotoxic levels of glutamate and other agents have employed only a limited number of antibodies to tau, and have not used the monoclonal antibodies that have

Abbreviations used: PHF, paired helical filament; HBSS, Hanks balanced salt-solution; LDH, lactate dehydrogenase; TBS, Tris-buffered saline.

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more recently been demonstrated to discriminate control adult brain tau from the more highly phosphorylated PHF-tau. It has only recently been realized that the phosphorylation state of a proportion of foetal tau is elevated compared with control adult brain tau, and that this fraction of foetal tau already resembles PHF-tau, as assessed by application of discriminatory antibodies [14–18]. In the present study we have used a panel of such monoclonal antibodies and taken account of the known higher level of phosphorylation of some of the foetal tau to re-examine the effects of toxic levels of glutamate, colchicine and β -amyloid on tau phosphorylation in rat cortical neurons in primary culture.

We report here that a proportion of tau in cultured rat cortical neurons normally has a higher level of phosphorylation than control adult brain tau, and is therefore similar to foetal tau *in vivo* and to PHF-tau from Alzheimer's-disease brain, and that dephosphorylation of this tau occurs after treatment with toxic levels of glutamate or colchicine, but before cell death, as assessed by release of lactate dehydrogenase. Concomitant with dephosphorylation, perikaryal tau immunoreactivity is increased, as assessed by immunocytochemical analyses of the cultures. These changes in tau phosphorylation in response to glutamate and colchicine treatment are opposite to what would be expected if they triggered the formation of PHF. Furthermore, we were unable to induce any changes in tau phosphorylation by exposing cultures to toxic doses of β -amyloid peptide in either the absence or the presence of glutamate, until there was obvious cell death, when the loss of tau immunoreactivity was most likely due to non-specific effects, e.g. release of lysosomal hydrolases.

MATERIALS AND METHODS

Antibodies

The preparation of the affinity-purified polyclonal antibody to human tau, 8073, and polyclonal antibody to bovine tau, B19, that recognize all tau isoforms independently of their phosphorylation states, have previously been described [8,19]. The mouse monoclonal antibody, Tau.1, that recognizes a dephosphorylated epitope in normal tau [20] was generously given by Dr. L. I. Binder (University of Alabama, Birmingham, AL, U.S.A.). The monoclonal antibody, AT8 [21], that recognizes a phosphorylated epitope (Ser-202) on PHF-tau and that corresponds to the Tau.1 epitope when unphosphorylated (Ser-199 and Ser-202) was a gift from Innogenetics Ltd., Zwijndrecht, Belgium. Antiserum TP70, raised to a synthetic C-terminal tau peptide, has been previously described [12]. The TP007 antiserum was raised to a synthetic peptide corresponding to the 16 most N-terminal residues of human tau that are present in all tau isoforms. RT97, 8D8, SMI31 and SMI310 are monoclonal antibodies that recognize phosphorylated neurofilament and PHF-tau [12,13,22–24].

Primary neuronal culture

Embryonic-rat (E17) cortices were dissected into Hanks balanced salt solution (HBSS; Gibco, Paisley, Scotland, U.K.), cleaned of meninges and chopped. HBSS was removed by aspiration and the tissue was incubated in HBSS containing 0.05% (w/v) trypsin (Sigma, Poole, Dorset, U.K.) at 37 °C for 15 min. The trypsin solution was then removed by aspiration and replaced with HBSS containing 8 mM $MgCl_2$, 0.002% (w/v) DNase, 10 mM Hepes and 5% (v/v) foetal-calf serum. The tissue was triturated in 2.5 ml of the same solution with a fire-polished glass Pasteur pipette. The cell suspension was diluted to 1×10^6 cells/ml in glutamine-free tissue-culture medium that consisted of Dulbecco's modified Eagle's medium mixed with Ham's F12

medium [DMEM:F12, 4:1 (v/v); Sigma] containing 100 μ g/ml transferrin, 60 μ M putrescine, 5 μ g/ml insulin, 20 nM progesterone, 30 nM sodium selenite, 50 units/ml penicillin, 50 μ g/ml streptomycin and 2% (v/v) heat-inactivated horse serum (all from Sigma) and plated into 25 cm² flasks (Marathon, London, U.K.) at a density of 3.2×10^5 cells/cm, or into 35 mm $\times$ 10 mm culture dishes (Marathon) at a density of 1.6×10^5 cells/cm that had been precoated with poly-L-lysine (10 μ g/ml) and laminin (10 μ g/ml) (both Sigma). Half of the tissue-culture medium was replaced after 5–7 days with DMEM:F12 (4:1, v/v) containing the supplements specified above, except without the addition of serum. This low-serum medium was sufficient to prevent non-neuronal cells proliferating. At 24 h before treatments, medium was fully replaced with the same serum-free media. Glutamate-, colchicine- and β -amyloid-induced degeneration of neuronal cultures was assessed by the release of lactate dehydrogenase (LDH) into the culture medium [25] (see below).

Experimental treatments

After 6, 8 or 10 days in culture, the cells were treated with 1 mM L-glutamate, 1 μ M colchicine or 250 nM okadaic acid (Sigma) for up to 6 h, or with 100 μ M β_{25-35} -amyloid peptide (BaChem, Torrance, CA, U.S.A.) for up to 24 h. The β_{25-35} -amyloid peptide was stored at room temperature in aqueous solution before addition to culture media; this 'aging' induced aggregation of the peptide, which is known to increase its neurotoxic properties. After treatments, portions of tissue-culture media were centrifuged at 15800 g_{av} , and supernatants were stored at –70 °C for determination of LDH. Cells from tissue-culture flasks were harvested into PBS and pelleted, whereas those in culture dishes were used for immunocytochemical analysis (see below). For total cell-lysate protein preparations, the cell pellet from each flask was resuspended in 0.5 ml of PBS, and a 50 μ l portion was added to SDS/PAGE sample buffer [62.5 mM Tris/HCl, pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.001% (w/v) Bromophenol Blue] for electrophoretic analysis (see below), heated for 5 min in a boiling-water bath and centrifuged at 15800 g_{av} for a further 5 min. The remaining cell suspension was centrifuged (15800 g_{av} , 1 min), and the pellet was resuspended in 40–100 μ l of Mes/NaCl buffer (100 mM Mes, 0.5 mM $MgCl_2$, 1 mM EGTA, 1 M NaCl, 2 mM dithiothreitol, 1 mM phenylmethanesulphonyl fluoride pH 6.5) and heated for 5 min in a boiling-water bath. The preparation was then centrifuged at 15800 g_{av} for 25 min. The supernatant containing the heat-stable fraction enriched in tau was retained; SDS/PAGE sample buffer was added, followed by a further 5 min incubation in a boiling-water bath.

For alkaline phosphatase treatment, cultured neurons were harvested into PBS, pelleted and resuspended in 100 μ l of Mes/NaCl buffer, heated for 5 min in a boiling-water bath and centrifuged at 15800 g_{av} for 25 min. The supernatant, enriched in tau, was removed and dialysed against 50 mM Tris, pH 8.3, containing 50 mM NaCl, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, 1 mM phenylmethanesulphonyl fluoride, 1 mg/ml pepstatin, 0.2 mg/ml aprotinin, 0.5 mg/ml leupeptin and 100 mM benzamidine (alkaline phosphatase reaction buffer). Alkaline phosphatase from calf intestinal mucosa (Boehringer Mannheim, Lewes, Sussex, U.K.) was added to portions of dialysed protein to give a final concentration of 400 units/ml and incubated overnight at 37 °C; for control samples, enzyme was omitted. After incubation, samples were heated in a boiling-water bath for 5 min, and SDS/PAGE sample buffer was added, followed by a further 5 min incubation period in a boiling-water bath.

Determination of neurotoxicity

LDH was assessed in culture media by using a LDH isoenzyme assay kit (Sigma) as an indication of neuronal cell damage [25]. Samples of media in incubation buffer were mixed with Cellstain reagent [10 parts of NAD^+ , 85 parts of lithium L(+)-lactate, 5 parts of Nitro Blue Tetrazolium and 0.1 part of phenazine methosulphate] and incubated at room temperature in the dark for up to 30 min. The A_{550} of the resulting colour was measured. The change in A_{550} measured in cultures treated with glutamate for 24 h, in which all neurons were dead, was taken as 100% LDH release. The means of six measurements from each treatment group were compared with the control group by Student's *t* test.

SDS/PAGE and Western blotting

Samples of total lysates of cultured cells and heat-stable preparations were separated on SDS/10% (w/v)-polyacrylamide gels in a discontinuous buffer system [26]. Separated proteins were then electrophoretically transferred on to nitrocellulose (Schleicher and Schuell, 0.45 μm pore size; Anderman, Kingston-on-Thames, Surrey, U.K.), blocked in Tris-buffered saline (TBS; 25 mM Tris, pH 8.0, 140 mM NaCl, 5 mM KCl) containing 0.2% (v/v) Tween 20 and 3% (w/v) dried skimmed milk (TBS-T-M) and incubated overnight in primary antibody diluted in TBS-T-M at 4 °C as described previously [9]. The filter was then washed (3×10 min in TBS containing 0.2% Tween 20; TBS-T) and incubated for 1 h in biotinylated donkey anti-rabbit or biotinylated sheep anti-mouse secondary antibody (Amersham International, Amersham, Bucks., U.K.; diluted 1:500 in TBS-T-M), followed by further washing in TBS-T and incubation for 1 h in streptavidin-alkaline phosphatase (Amersham; diluted 1:3000 in TBS-T-M). After final washes in TBS-T, blots were developed by using an alkaline phosphatase conjugate substrate kit (Bio-Rad, Hemel Hempstead, Herts., U.K.). Before separation by SDS/PAGE, the protein content of each sample was determined, by using a dot-blot stained with Coomassie Blue and so that loading on to the electrophoresis gels could be normalized for total protein.

Immunocytochemical labelling of cultures

Cultures were fixed for 20 min with 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, washed in the same phosphate buffer and stored at 4 °C in TBS. These tissue-culture samples were labelled by the peroxidase-antiperoxidase (PAP) method. Cultures were first treated for 30 min with TBS containing 0.2% Triton X-100 (TBS-TX), incubated in blocking solution [TBS-TX containing 10% (v/v) normal goat serum], and then treated for 16 h at 20 °C with the primary antibody diluted in TBS-TX containing 1% normal goat serum. The cultures were then incubated with a goat anti-rabbit or goat anti-mouse antibody (Nordic, Turnhout, Belgium; diluted 1:30 in TBS-TX containing 1% normal goat serum) followed by a rabbit or mouse PAP complex (Nordic; diluted 1:300 in TBS-TX containing 1% normal goat serum). The peroxidase activity was revealed by using diaminobenzidine as chromogen.

RESULTS

Glutamate-induced dephosphorylation of tau in primary rat cortical neuronal cultures

Initially, the effects of the excitatory amino acid glutamate on the phosphorylation state of foetal tau were studied in 10-day-old

Figure 1 Time-course effect of glutamate and colchicine on tau in primary neuronal cultures

Western blots of total cell-lysate proteins from primary 10-day-old rat cortical neuronal cultures treated with (a) glutamate (1 mM) and (b) colchicine (1 μM) for 0 min (lane 1), 5 min (lane 2), 30 min (lane 3), 1 h (lane 4), 3 h (lane 5) and 6 h (lane 6). The blots were probed with 8073 or Tau.1, as indicated. Arrows show the positions of the major tau broad band/doublet, and arrowheads indicate the positions of the two intact less phosphorylated and faster-migrating tau species. Numbers to the right of the Figures represent approximate molecular masses (kDa).

primary rat cortical neuronal cultures. Figure 1 shows Western blots of total cell-lysate proteins from cultures treated with (Figure 1a) glutamate (1 mM) and (Figure 1b) colchicine (1 μM) for up to 6 h and labelled with an affinity-purified polyclonal antibody to human tau, 8073, and the monoclonal antibody, Tau.1, as indicated. As we described previously for foetal tau *in vivo*, the major tau species run either as a broad band or as a closely migrating and poorly resolved doublet of approx. 48 kDa (arrows), that is labelled by the polyclonal antibody, but only the faster-migrating species of the doublet (or portion of the broad band) was labelled by Tau.1 [14]. However, after incubation for 30 min with glutamate, two lower-molecular-mass bands (arrowheads) were recognized by 8073 and Tau.1 (Figure 1a, lanes 3), which became more intense with prolonged glutamate incubation (Figure 1a, lanes 4–6). A similar pattern of tau immunoreactivity was observed after colchicine treatment (Figure 1b), in that the same faster-migrating tau species (arrowheads) became more intensely labelled by 8073 and Tau.1 after incubation with colchicine for 30 min (Figure 1b, lanes 3), and became more intensely labelled with prolonged exposure (Figure 1b, lanes 4–6).

Over the course of glutamate treatment, there was a 22% increase in LDH release above control levels after 6 h, but at shorter times up to 3 h, there was no significant increase in LDH release, even though there was already a marked change in tau mobility and immunoreactivity. By 12 h of glutamate treatment there was a near-maximal release of LDH of 94% above control levels. This pattern of LDH release over time during an excitotoxic response is similar to that previously described [27]. We

Figure 2 Glutamate-induced dephosphorylation of tau in primary neuronal cultures

Western blots of total cell-lysate proteins from primary 10-day-old rat cortical neuronal cultures treated with glutamate (1 mM) for 0 min (lane 1) or 6 h (lane 2), and incubated with (a) 8073, (b) Tau.1, (c) TP70, (d) TP007, (e) AT8, (f) 8D8, (g) RT97, (h) SMI31 and (i) SMI310, as indicated. Arrows indicate the positions of the major tau broad band/doublet, and arrowheads indicate the positions of the two main less phosphorylated and faster-migrating intact tau species. A minor higher-molecular-mass tau species (approx. 57 kDa) seen with TP70, TP007 and 8D8 is probably a longer tau isoform (see the Results section). Asterisk indicates the higher-molecular-mass band that corresponds to the neurofilament protein NF-H. Numbers to the right of the Figure represent approximate molecular masses (kDa).

also observed a similar pattern of LDH release after colchicine treatment of the primary neuronal cultures (results not shown).

Figure 2 shows Western blots of total cell-lysate protein from 10-day-old cultures that were treated with glutamate (1 mM) for zero (control) or 6 h, and have been probed with a panel of antibodies. TP70 (Figure 2c) and TP007 (Figure 2d) (polyclonal antibodies that recognize the C- and N-termini of tau respectively) both labelled the initial tau broad band/doublet (arrows) and the same strongly immunoreactive faster-migrating species (arrowheads) as recognized by 8073 (Figure 2a) and Tau.1 (Figure 2b) that appear after glutamate treatment of the cultures. On some blots, an additional higher-molecular-mass tau species of approx. 55–57 kDa was recognized by the polyclonal antibodies TP70 and TP007 and the monoclonal neurofilament antibody 8D8. This probably corresponds to a longer tau isoform and can also be detected in 3-day-postnatal rat brain [14]. The labelling of the faster-migrating tau species (arrowheads) by antibodies to both extremities of the tau polypeptide demonstrates that these principal faster-migrating species are not the products of proteolysis, but rather are due to dephosphorylation of tau; these species are also hardly labelled, if at all, by the phosphate-dependent monoclonal antibodies (Figures 2e–2i). Similar dephosphorylation *in vitro* of neuronal tau in cell lysates by alkaline phosphatase in the presence of a cocktail of protease inhibitors also demonstrated that the faster-migrating species were the product of dephosphorylation and not proteolysis (see below and Figure 4). The additional and apparently smaller species than those indicated by arrowheads that were present after glutamate treatment and recognized by Tau.1 (Figure 2b) and TP007 (Figure 2d), are probably tau degradation products, because they were not recognized by the C-terminal antibody TP70 (Figure 2c).

The monoclonal antibodies AT8, 8D8, RT97, SMI31 and SMI310 (Figures 2e–2i respectively), all of which recognize phosphorylated tau epitopes, and which label only the slower-migrating species of the broad band/doublet of tau [14], showed a decrease in intensity of labelling of this tau species after glutamate treatment of cultures for 6 h and, indeed, the labelling by AT8 was abolished. It was with these phosphate-dependent monoclonal antibodies that two differently phosphorylated

species of tau were most clearly observed, since in untreated cultures they consistently labelled a narrow and the most slow-migrating fraction of the broad band/doublet of tau (Figures 2e–2i). Conversely, Tau.1 showed an increase in staining intensity of the faster-migrating component of the broad band/doublet after 6 h of glutamate treatment (Figure 2b). These results suggest that a significant proportion of the tau in primary rat cortical cultures is normally highly phosphorylated and becomes dephosphorylated after glutamate treatment. Although neuronal death occurs, since LDH is released, this process is gradual, as has been demonstrated by others [27–30], and the dephosphorylation of tau commences before this lytic stage (Figure 1), but is more evident after 6 h of treatment.

The monoclonal antibodies 8D8, RT97, SMI31 and SMI310 (which are all neurofilament antibodies) all recognized a high-molecular-mass band, corresponding to neurofilament protein NF-H (Figure 2, asterisk), in both control and treated samples. After glutamate stimulation, a second high-molecular-mass band was recognized by all of these neurofilament antibodies that may correspond to neurofilament protein NF-M or a degradation product of NF-H. In addition, these neurofilament monoclonal antibodies variably labelled to different intensities several other species throughout the molecular-mass range that may be degradation products of neurofilaments. On the other hand, as all of these antibodies selectively recognize phosphorylated epitopes, glutamate treatment of the cultures, conversely to its effects on tau, may induce phosphorylation of some proteins that are then cross-reactive with monoclonal antibodies against phosphorylated NF-H. Indeed, 8D8 staining of a band in the NF-M region is strongly increased by glutamate treatment, and since the control sample does not contain a longer species of comparable immunoreactivity, it would appear that this molecular-mass band is most likely due to an increase in neurofilament phosphorylation.

Colchicine-induced dephosphorylation of tau in primary rat cortical neuronal cultures

Colchicine treatment of both 6- and 10-day-old rat primary cortical cultures had a similar effect to glutamate on the

Figure 3 Colchicine-induced dephosphorylation of tau in primary neuronal cultures

(a–f) Western blots of total cell-lysate proteins from control primary 6-day-old rat cortical cultures (lanes 1) and 6-day-old cultures treated with colchicine ($1 \mu\text{M}$) for 6 h (lanes 2), and incubated with (a) 8073, (b) Tau.1, (c) TP70, (d) TP007, (e) AT8 and (f) 8D8; (g) and (h) are Western blots of total cell-lysate proteins extracted from 10-day cultures incubated with (g) AT8 and (h) 8D8. Arrows indicate the positions of the major tau broad band/doublet, and arrowheads indicate the positions of the two intact less phosphorylated and faster-migrating tau species. As in Figure 2, a minor tau-positive species (approx. 57 kDa) indicates the presence of a longer tau isoform; the presence of this species is most marked with TP70. TP70 probably also labels MAP2C (asterisk), and this too is shifted to faster-migrating forms after colchicine treatment. Numbers to the right of the Figure represent approximate molecular masses (kDa).

Figure 4 Dephosphorylation *in vitro* of tau extracted from primary neuronal cultures

Western blots of control heat-stable proteins from primary rat cortical cultures (lane 1) and heat-stable proteins from primary cortical cultures treated with alkaline phosphatase for 16 h at 37°C (lane 2), and probed with (a) Tau.1, (b) TP70, (c) TP007 and (d) AT8. Arrowhead indicates the position of the less phosphorylated and faster-migrating tau species. Numbers to the right of the Figure represent approximate molecular masses (kDa).

phosphorylation state of tau, as assessed both by Western-blot analysis (Figure 3) and immunocytochemically (results not shown). The polyclonal antibody 8073 showed the generation of increased amounts of lower-molecular-mass species (arrowheads) on Western blots (Figure 3a), after treatment of 6-day cortical cultures with $1 \mu\text{M}$ colchicine for 6 h. Tau.1 showed the appearance of two faster-migrating tau species (arrowheads) after colchicine treatment (Figure 3b), whereas AT8 failed to label tau after the same treatment in both 6- and 10-day cultures (Figures 3e and 3g respectively). After treatment with colchicine, the monoclonal anti-neurofilament antibody 8D8 showed a modest decrease in intensity of labelling on 6-day cultures and abolition of labelling of 10-day cultures (Figures 3f and 3h respectively). Again, these changes were not the result of tau proteolytic degradation, since the principal faster-migrating bands (arrowheads) were both labelled by the antibodies TP70 and TP007 (C-

and N-terminal anti-tau polyclonal antibodies, respectively) (Figures 3c and 3d respectively), although additional weakly tau-positive bands migrating faster than those indicated by arrowheads probably are degradation products. The higher-molecular-mass band recognized by the polyclonal antibody TP70 (asterisk) is probably due to cross-reactivity with the microtubule-associated protein MAP2C, which is also shifted to a faster-migrating species after colchicine treatment, tau and MAP2C having a high degree of similarity at their C-termini [31]. The higher-molecular-mass tau species also recognized by TP70 (approx. 57 kDa) is referred to above.

Dephosphorylation *in vitro* of tau from primary rat cortical neuronal cultures

In order to confirm further that the observed effects of glutamate and colchicine on tau immunoreactivity were indeed due to dephosphorylation of tau, a heat-stable protein preparation from 10-day primary cortical cultures was treated with alkaline phosphatase in the presence of a cocktail of protease inhibitors (see the Materials and methods section). After enzyme treatment, a proportion of the tau had an increased electrophoretic mobility (Figures 4a–4c, arrowhead) and showed a total loss of labelling with the phosphorylation-dependent antibody AT8 (Figure 4d). This dephosphorylation was similar to, although not identical with, that seen after glutamate and colchicine treatments of the neuronal cultures. Not all of the tau was dephosphorylated by alkaline phosphatase treatment *in vitro*, and we have found it difficult to achieve complete dephosphorylation. The N- and C-terminal antibodies TP70 and TP007 also labelled this dephosphorylated tau (Figures 4b and 4c), demonstrating that this faster-migrating species was the result of dephosphorylation and not degradation of tau. Tau.1 also labelled small amounts of additional faster-migrating species after alkaline phosphatase treatment, which probably were the result of proteolysis (Figure 4a).

Immunocytochemistry of primary rat cortical neuronal cultures

The effects of glutamate and colchicine treatments on the distribution and the phosphorylation status of tau in cultured rat

Figure 5 Glutamate-induced dephosphorylation of tau in primary neuronal cultures *in situ*

Immunocytochemical staining of control (a, c and e) and glutamate-treated (1 mM; b, d and f) 10-day-old primary rat cortical neurons with polyclonal anti-tau antibody B19 (a and b), or monoclonal antibodies Tau.1 (c and d) and AT8 (e and f). Glutamate treatment induced an increase in B19 perikaryal immunoreactivity in a subpopulation of neurons (arrows in b); many neurons showed an increase in Tau.1 perikaryal immunoreactivity and a decrease in AT8 staining. Scale bar, 50 μ m.

cortical neurons was investigated by immunocytochemical labelling *in situ* with polyclonal antiserum B19 and the monoclonal antibodies AT8 and Tau.1.

In 10-day-old control cortical cultures, the polyclonal antibody B19 labelled axons and neuronal cell bodies (Figure 5a); the Tau.1 monoclonal antibody labelled axons with variable intensity, some long axons being strongly labelled, but this antibody labelled the perikarya only weakly (Figure 5c). AT8 displayed labelling very similar to that of B19, i.e. a labelling of cell bodies and axons (Figure 5e). A significant number of neurons also showed a labelling of dendrites or the proximal part of dendrites, with both B19 and AT8 (Figures 5a and 5e). Glutamate treatment (1 mM for 6 h) of 10-day-old cultured cortical neurons induced marked changes in the tau immunoreactivity. The polyclonal B19 antibody still labelled axons and perikarya, but the tau immunoreactivity in perikarya was clearly increased in a significant proportion of neurons (Figure 5b). Tau.1 strongly labelled many neuronal perikarya (Figure 5d), while still demonstrating immunoreactivity with axons. Contrary to this, AT8

immunoreactivity was strongly decreased throughout glutamate-treated neurons (Figure 5f).

Immunocytochemical labelling of 6-day-old control cortical neurons with the polyclonal antibody B19 and monoclonal antibodies Tau.1 and AT8 was similar to that described above for the 10-day-old control cultures (results not shown). In colchicine-treated (1 μ M for 6 h) 6-day-old neuronal cultures, the change in immunoreactivity with the polyclonal antibody B19 and monoclonal antibodies Tau.1 and AT8 was similar to that seen after glutamate treatment, in that B19 and Tau.1 both showed an increase in labelling of many neuronal perikarya and AT8 showed a decrease in neuronal immunoreactivity in a significant proportion of neurons (results not shown).

Effect of okadaic acid on glutamate-induced tau dephosphorylation

In order to investigate potential phosphatase involvement in the glutamate-induced dephosphorylation of neuronal tau, we

Figure 6 Effects of okadaic acid on glutamate-induced tau dephosphorylation

Western blots of total cell-lysate proteins from untreated 10-day-old rat cortical neuronal cultures (**a**, **b** and **c**, lanes 1) or treated with okadaic acid (250 nM) for 10 min (**a** and **b**, lanes 2), 30 min (**a** and **b**, lanes 3), 1 h (**a** and **b**, lanes 4) and 6 h (**a** and **b**, lanes 5), or okadaic acid (250 nM) and glutamate (1 mM) for 10 min (**a**, lane 6, and **c**, lane 2), 30 min (**a**, lane 7, and **c**, lane 3), 1 h (**a**, lane 8, and **c**, lane 4) and 6 h (**a**, lane 9, and **c**, lane 5) and probed with (**a**) TP70, (**b**) Tau.1 or (**c**) 8D8. Numbers to the right of the Figure represent approximate molecular masses (kDa).

studied the effects of okadaic acid on this process. However, we found that 250 nM okadaic acid alone further slightly decreased the mobility of tau after 1 h of treatment, as assessed with the polyclonal antibody TP70 (Figure 6a, lane 4), and the intensity of staining of tau by monoclonal antibody Tau.1 after 1 h of okadaic acid treatment was decreased, indicating increased phosphorylation at Ser-199/202 (Figure 6b). Both of these changes are consistent with additional overall phosphorylation of the tau.

When cells were treated with both okadaic acid and glutamate, then the two effects of each agent occurred together. Thus, the two faster-migrating tau species were generated and the slowest-migrating tau band was apparently even further decreased in mobility (Figure 6a, compare lane 1 with lanes 6–9). However, the staining by monoclonal antibody 8D8 was decreased (Figure 6c, lane 5). This implies that the effects of okadaic acid treatment are complex, in that it inhibits some phosphatase activity to cause a further decrease in mobility, but it does not inhibit the phosphatase activity responsible for dephosphorylating the 8D8 epitope in these cultures.

β -amyloid failed to induce PHF-like phosphorylation of tau in primary rat cortical neuronal cultures

Treatment of 8-day-old primary rat cortical neuronal cultures with aggregated β_{25-35} -amyloid peptide (100 μ M for 24 h) was associated with marked toxicity (maximal LDH release and histological results not shown). The polyclonal antibodies 8073, TP70 and TP007 all labelled tau extracted from cultures treated with β_{25-35} -amyloid peptide less intensely than control tau (Figures 7a, 7b and 7c, respectively), which is probably due to general protein degradation associated with cell death. These antibodies also recognized faster-migrating immunoreactive species after treatment of the cultures with β_{25-35} -amyloid peptide (Figures 7a, 7b and 7c), some of which are possibly the result of dephosphorylation of tau, as they are labelled by all three polyclonal antibodies, whereas the smaller species are variably recognized and are probably the result of proteolysis. The phosphorylation-dependent monoclonal antibodies AT8 and

Figure 7 Effects of β_{25-35} -amyloid peptide on primary neuronal cultures

Western blots of heat-stable proteins from 8-day-old control primary rat cortical cultures (lane 1) and cultures treated with aggregated β_{25-35} -amyloid peptide (100 μ M) for 24 h (lane 2) and probed with (**a**) 8073, (**b**) TP70, (**c**) TP007, (**d**) AT8 and (**e**) 8D8. Arrows indicate the positions of the major tau broad band/doublet. As in Figure 2, a minor tau-positive species (approx. 57 kDa) indicates the presence of a longer tau isoform; the presence of this species is most marked with TP70 and 8D8. Numbers to the right of the Figure represent approximate molecular masses (kDa).

8D8 also did not recognize these faster-migrating bands, but labelled the initial broad tau band/doublet less intensely after β_{25-35} -amyloid peptide treatment (Figures 7d and 7e respectively) of the cultures. However, this decrease in immunoreactivity with the phosphate-dependent antibodies was not obviously greater than that observed with the polyclonal antibodies, demonstrating that tau degradation rather than dephosphorylation is the predominant response to treatment with β_{25-35} -amyloid peptide.

We have tried many different experimental paradigms in order to investigate the effect of β_{25-35} -amyloid on tau phosphorylation in primary neuronal cultures, and the results shown in Figure 7 are typical of the data obtained. These include low- and high-density hippocampal and cortical cultures which have been grown for up to 31 days and treated both acutely and chronically with various concentrations of peptide, in either the presence or the absence of glutamate. Although we have consistently been able to induce toxicity, we have never seen any evidence of an increase in tau phosphorylation, such as might be indicative of PHF-like changes in the neuronal cytoskeleton.

DISCUSSION

We and others have demonstrated that a proportion of foetal tau in rat and human brain is in an elevated state of phosphorylation compared with adult brain tau. Thus, it has been shown by Western-blot analysis, using antibodies that discriminate between phosphorylated and non-phosphorylated epitopes, that foetal tau is a mixture of at least two major species, one of which is more highly phosphorylated than the other [14–17]. Here we have shown that tau extracted from primary cultures of rat cortical neurons appears to be very similar to that extracted directly from foetal brain, and these two phosphorylation states of tau persist in the cultured neurons for at least 10 days.

In the present study we have demonstrated that treatment of primary neuronal cultures with glutamate or colchicine results in dephosphorylation of the more heavily phosphorylated tau species. This dephosphorylation is both progressive and a phenomenon that occurs during the process of neuronal degeneration, but before significant cell lysis has occurred. One laboratory has reported that glutamate treatment of neurones in

culture induces an increase in tau phosphorylation [32,33], but these studies were over short periods of treatment, and we have been unable to confirm these findings, and the reason for this discrepancy is as yet unclear.

Although the faster-migrating tau component of the broad band/doublet present initially in the cultured neurons, and which is labelled by Tau.1 but not the phosphate-dependent monoclonal antibodies, is clearly less phosphorylated than the slightly slower migrating species, it nevertheless is phosphorylated, since treatment of the cultures with glutamate resulted in the appearance of additional and faster-migrating species (arrowheads in Figures 1–3). This was confirmed by the treatment *in vitro* with alkaline phosphatase of heat-stable tau extracted from the cultures. It is therefore likely that this faster-migrating species of foetal tau is phosphorylated at sites other than those recognized by the panel of available monoclonal antibodies, all of which recognize phosphorylated serine/threonine residues followed by proline, i.e. SP or TP motifs. These extra phosphorylation sites may reside in the C-terminal half of the molecule, since we and others have shown that phosphorylation *in vitro* of the C-terminal half of recombinant tau by cyclic AMP-dependent protein kinase or Ca^{2+} /calmodulin-dependent protein kinase, probably at sites other than SP or TP residues, results in a significant upward shift in mobility of tau [34–39].

The dephosphorylation of foetal tau induced by glutamate or colchicine was incomplete, because not all the tau shifted to the fastest-migrating form. Nevertheless, the epitopes recognized by the phosphate-dependent monoclonal antibodies were in some experiments completely dephosphorylated. The mechanism by which glutamate and colchicine induce a dephosphorylation of tau in primary neuronal cultures has yet to be elucidated, but, since both these agents give rise to an increase in perikaryal tau immunoreactivity, it is possible that they share a similar mechanism that might involve a decrease in the number of polymerized microtubules. Glutamate could act by increasing intracellular Ca^{2+} levels, thus leading to microtubule destabilization and consequently the release of tubulin-bound tau [3,32,40]. Direct destabilization of microtubules by colchicine, independently of Ca^{2+} influx, might have the same effect. Phosphorylation appears to regulate the tau–microtubule interaction [41–44], and so, if there is a shift from polymerized to unpolymerized tubulin, then dephosphorylation of tau may be a compensatory mechanism to promote microtubule assembly after neurotoxic insult. Sygowski et al. [45] also demonstrated that colchicine treatment of NIH 3T3 fibroblasts expressing transfected tau resulted in the tau becoming less phosphorylated, although not all microtubule-destabilizing agents produced this effect.

The regulation of tau phosphorylation is probably complex, since experiments with okadaic acid treatment of neuronal cultures produced a slight decrease in electrophoretic mobility of tau, and this was still observed when the cultures were treated with a mixture of glutamate and okadaic acid, although under the latter conditions staining by the monoclonal antibody 8D8 was decreased markedly. This implies that inhibition of protein phosphatases PP1 and PP2A by okadaic acid causes an electrophoretic-mobility shift, but that these phosphatases do not act on the 8D8 epitope. Indeed, studies *in vitro* have demonstrated that protein phosphatases PP1, PP2A, PP2B and PP2C can all dephosphorylate tau, but with differential potencies towards individual sites [46–48].

Our immunocytochemical results confirm the findings of Mattson and colleagues [3,40] that both glutamate and colchicine treatment of neurons in primary culture results in increased perikaryal immunoreactivity of tau, but our data also show that this relocation of tau from axons to neuronal cell bodies is

accompanied by a decrease in phosphorylation of the protein, consistent with the Western blot analysis (Figures 1, 2 and 3). These immunocytochemical changes in the presence of colchicine and glutamate occurred during the course of neuronal degeneration, but were apparently not the consequence of cell death.

This increase in perikaryal tau immunoreactivity in primary neurons treated with glutamate, colchicine or glutamate, in conjunction with β -amyloid peptides, has previously been suggested to mimic changes observed in neurons containing PHF in Alzheimer's disease, and it was proposed that one mechanism by which β -amyloid is neurotoxic is by acting synergistically with glutamate [2,49,50]. However, the dephosphorylation of tau observed in glutamate- and colchicine-treated primary neurons in the present study is opposite to the increase in phosphorylation that is associated with the conversion of adult brain tau into PHF-tau. We found, however, that β_{25-35} -amyloid alone induced no obvious change in the phosphorylation state of tau in primary neuronal cultures, and we failed to observe any synergistic effect of β_{25-35} -amyloid and glutamate on tau phosphorylation. Using the available antibodies, on no occasion did we observe an increase in the proportion of tau in an elevated state of phosphorylation after β_{25-35} -amyloid treatment of neuronal cultures, even when β_{25-35} -amyloid-induced toxicity was clearly apparent.

In conclusion, although the immunocytochemical changes seen in primary neurons treated with glutamate and colchicine superficially mimic those seen in neurons containing PHF in Alzheimer's disease, i.e. an accumulation of tau in neuronal perikarya [51], this redistribution is associated with a dephosphorylation rather than increased phosphorylation of tau. This dephosphorylation is associated with neuronal degeneration (as assessed by release of LDH), but commences before there is any significant cell lysis. The pattern of phosphorylation changes is thus opposite to that associated with PHF formation in Alzheimer's disease. However, since primary rat cortical neurons do contain a significant proportion of tau in an elevated state of phosphorylation, they thus represent a valuable cellular model for investigating those mechanisms that regulate tau phosphorylation during development, and for identifying candidate phosphatases or kinases that may, nevertheless, be involved in tau phosphorylation in the pathogenesis of Alzheimer's disease.

We thank Dr. Jim Cohen for advice on preparing primary neuronal cultures and Innogenetics for providing the AT8 antibody. This work was supported by the British Medical Research Council, the Wellcome Trust, Glaxo Research and Development Ltd., the British Council, NATO, the Belgian F.R.S.H. (3.450.91) and the Fonds de Recherche Divvy.

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