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Cell cycle markers in the hippocampus in Alzheimer's disease

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Abstract Using immunohistochemistry we have analysed the nuclear expression of cyclins A, B, D, and E in neurones in the hippocampi of control subjects and patients suffering from various neurodegenerative disorders including Alzheimer's disease (AD). Cyclins A and D could not be detected but varying degrees of cyclin E expression were found in all patient groups including control subjects. Cyclin B expression was not detected in control subjects but it was expressed in the subiculum, dentate gyrus and CA1 region in patients with AD-type pathology and in the CA2 region and the dentate gyrus of cases of Pick's disease. These reults suggest that some neurones may have re-entered the cell cycle. The expression of cyclin E without cyclin A expression may indicate an arrest in G_1 with the possibility of re-differentiation and exit from G_1 to G_0. The expression pattern of cyclin E indicates that re-entry into the cell cycle is possible even in control patients, but it is accentuated in patients with AD-related pathology. However, cyclin B was only expressed in AD patients and occurred in areas that were severely affected by pathology. Neurones with cyclin B-reactive nuclei in AD were AT8 positive but did not contain fully developed tangles. In neurones, where cyclin B is expressed, it would appear that the G_1/S checkpoint has been bypassed and that the cell cycle is arrested in G_2. It is proposed that these neurones do not have the opportunity for subsequent re-differentiation. Since factors known to be present in G_2 seem to be responsible for microtubule destabilisation and hyperphosphorylation of tau we hypothesise that cell cycle disturbances may be important in the pathogenesis of AD.

Key words Cell cycle · Alzheimer's disease · Down's syndrome

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Introduction

The classical view about neurones of the adult central nervous system is that they are resting cells that are unable to re-enter the cell cycle and undergo cell division [7, 8, 14]. However, there are several studies indicating that mature neurones are able to re-enter the cell cycle in vitro [15] and, depending on the environmental signals provided, they may either become committed to the cell cycle and undergo cell division or die via an apoptotic pathway [15]. Generally the genetically programmed differentiated phenotype is maintained by specific environmental signals that are unique for each specific cell type. When environmental signals change in the appropriate direction cells leave the resting phase and re-enter the cell cycle [5, 13]. From extensive cancer-related studies it is known that the main regulators of the cell cycle are the cyclin-cyclin-dependent kinase (cdk) complexes. The expression/activation of these complexes triggers the transition of the cell through the checkpoints where cell cycle regulation occurs. There are several checkpoints in G_1 and G_2 [7]. Before reaching a late G_1 checkpoint cells can return to G_0 [7]. After passing the restriction point in late G_1 cells are committed to the cell cycle even in the absence of growth factors [7, 13]. The best known cyclins are cyclins D, E, A and B. Cyclin D expression is inducible by growth factors at the G_0/G_1 transition [8, 16, 26, 28] and it is rapidly degraded after growth factor withdrawal [13, 16, 26, 28]. There is no oscillation of cyclin D levels during the cell cycle [13, 16, 26, 28]. Its expression is also associated

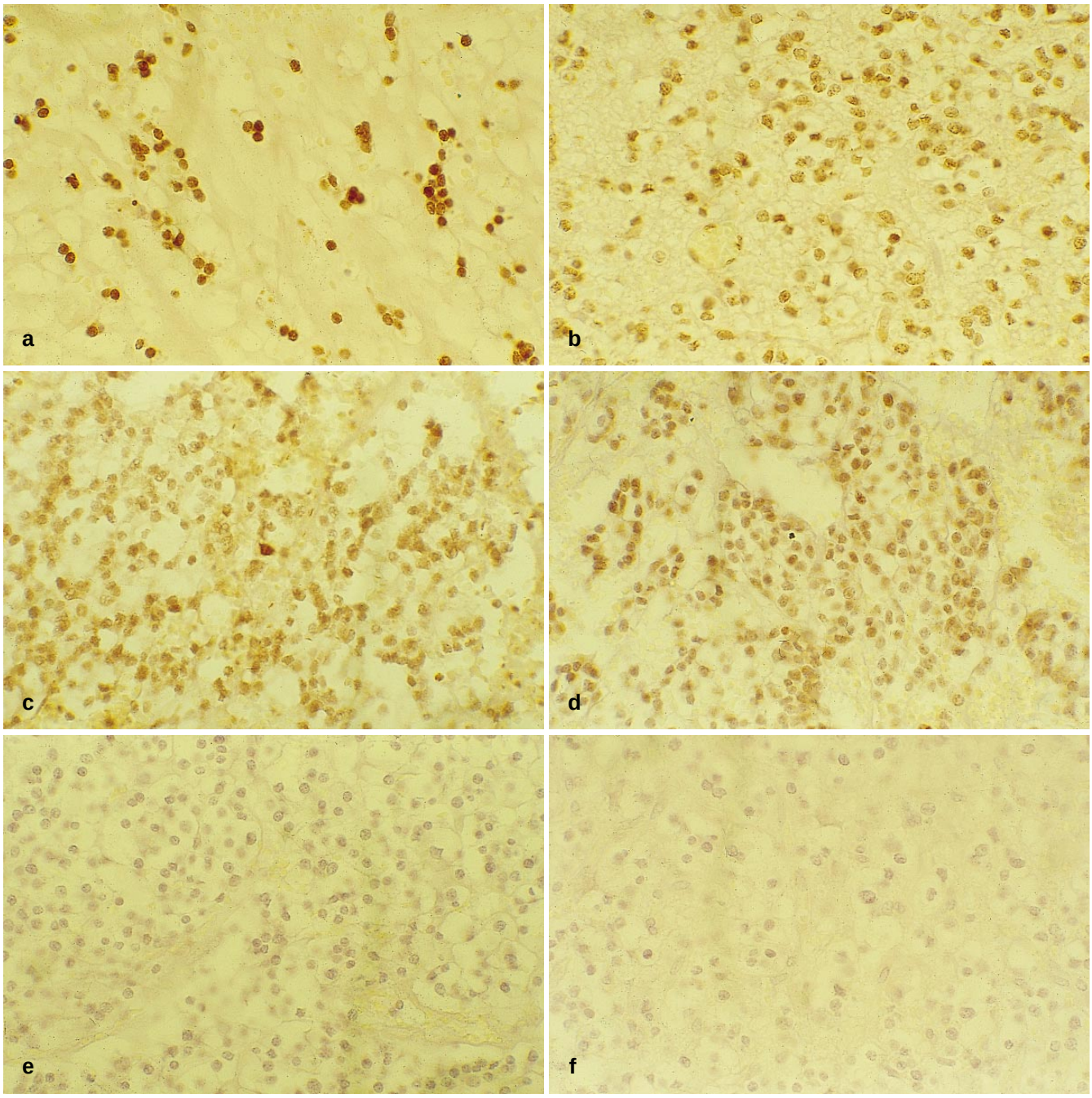

Fig. 1 a–f Cyclin expression in tumour tissue. **a** Cyclin A expression in glioma; **b** cyclin D expression in glioma; **c** cyclin B expression in glioma; **d** cyclin E expression in glioma; **e** cyclin B labelling in glioma after peptide absorption; **f** cyclin E labelling in glioma after peptide absorption

with programmed cell death in postmitotic neurones [11]. Cyclin E production starts at the beginning of G_1 and reaches a maximum at the G_1/S transition [8, 13, 26, 28]. It is degraded at the beginning of the S phase [13, 26, 28]. It is known to be associated with cdk2 and the activity of cyclin E/cdk2 complex is needed for the G_1/S transition [13, 16, 25, 28]. Cyclin E is also believed to be important for the activation of DNA synthesis [16, 28]. The overexpression of cyclin E may override growth inhibitory signals [16], while deactivation leads to G_1 arrest [13]. Cyclin A accumulates in the nucleus [9, 10, 22] as soon as it is synthesised in late G_1. Its levels reach a maximum in the S phase [5, 7, 10, 13] and it is rapidly degraded in the M phase [7, 10, 22]. Inhibition or ablation of cyclin A leads to G_2 arrest [22, 26] with the premature appearance of cyclin B [10] while overexpression of cyclin A leads to accelerated G_1/S transition [9]. Cyclin B is detectable in the cytoplasm during interphase [5, 10, 16, 17, 26] and enters the nucleus only at the beginning of mitosis [5, 8, 10, 17, 26]. Activation of this cyclin is needed for the G_2/M transition in the absence of protein synthesis [13, 17]. If this cyclin is not degraded at the end of mitosis cells can-

not leave mitosis [5, 7, 13, 26]. DNA damage or lack of DNA replication inhibit the activation of cyclin B and cause G_2 arrest [10, 13, 16, 26].

De-regulation of the cell cycle at the checkpoints enumerated above may result in apoptosis and a wide variety of apoptotic agents require molecules synthesised in late G_1 or the S phase of the cell cycle to trigger apoptosis [9]. It is believed that the expression of positive regulators of the cell cycle in an environment that is unable to support cell division results in cell death via an apoptotic pathway [11]. There are multiple possible starting points for apoptosis during the cell cycle involving the G_1/S transition or the G_2/M transition [24].

Cell cycle-related proteins and kinases involved in cell cycle regulation may also be involved in the control of cytoskeletal dynamics and organisation [17]. Thus, cyclin B but not cyclin A, produces microtubule shrinkage and destabilisation [22], while microtubule depolymerisation is involved in providing the signal for the synthesis of DNA [14]. In addition, mitogen-activated kinases, glycogen synthase kinase 3β (GSK-3β) and cdk5 involved in cell cycle regulation, are capable of phosphorylating tau in vivo [23]. In vitro cdk2/cyclin A can phosphorylate tau into Alzheimer's disease (AD)-type tau [4]. Cdk5 is physically associated with microtubules and is also capable of transforming tau into an AD-type state by phosphorylation [3, 4, 17]. It is also interesting that in vitro experiments indicate that the microtubule-associated protein tau is hyperphosphorylated in an AD-like manner during mitosis [27].

Since in vitro studies indicate that a synthetic β-amyloid (Aβ) homologue (β1-42) stimulates the proliferation and morphological transformation of microglia [2], while the processing of the amyloid precursor protein (APP) into amyloidogenic fragments is associated with the G_2 phase of the cell cycle [29] and the link between Aβ aggregates and signal transduction pathways is implicated in diverse cell functions including neurite outgrowth, cell cycle control and apoptosis [33], we thought it worthwhile looking for in vivo evidence of cell cycle de-regulation in AD and related disorders. Our initial study of Ki-67 expression indicated that in neurodegenerative disorders hippocampal neurones may re-enter the cell cycle [21]. In this report we examine the expression of cyclins D, E, A and B in the hippocampus in relation to AD-related and other neurodegenerative pathology.

Materials and methods

Hippocampi from 31 patients were included in the study. Brain tissue was fixed in 10% formalin for at least 4 weeks. Serial sections 10 μm thick, were cut and immunolabelled for cyclins A, B, D and E (rabbit polyclonal antibodies from Santa-Cruz). Adjacent sections were labelled with AT8 antibody (mouse monoclonal from Innogenetics) to show the cytoskeleton-related AD-type changes [12]. Immunolabelling was carried out using the simple avidin-biotin-peroxidase complex (ABC) technique for immunocytochemistry. For the AT8 labelling no special pretreatment was required, whereas before labelling with anti-cyclin antibodies sections were microwaved in citrate buffer (0.01 M, pH 6.0). We used the cyclin A and AT8 antibodies at 1:50 dilution, while the other cyclin (D, E and B) antibodies were used at 1:200 dilution. The secondary antibody (anti-rabbit and anti-mouse, Amersham) was used at 1:200 dilution. Streptavidin-horseradish peroxidase (HRP, DAKO) was used at a 1:400. The chromogene applied was the metal-enhanced diaminobenzidine (Pierce-Warrin). Double-labelling experiments with AT8 and cyclin B antibodies were carried out in some of our patients to see the possible co-localisation of the two proteins. For this purpose

Table 1 Patient groups analysed in this study (*AD* Alzheimer's disease)

Patient group	Diagnosis	No. of patients	Age at death in years mean (range)
C	Control	3	39.13 (18, 20, 79.4)
DS	Down's syndrome patients without any AD pathology	2	13 (13, 13)
Pre AD		16	
C/preAD	Some AD-related pathology insufficient for the pathological diagnosis of AD	7	85.3 (80.7 – 90.6)
DS/preAD	DS patients with AD-related pathology insufficient for the pathological diagnosis of AD	2	41 (40, 42)
Epil/preAD	Epileptic patients with AD-related pathology insufficient for the pathological diagnosis of AD	2	74 (71, 77)
preAD+	Parkinson's disease patients with AD-related pathology insufficient for the pathological diagnosis of AD	5	80.7 (61 – 93.3)
AD		8	
AD	AD-related pathology sufficient for the pathological diagnosis of AD	5	80.1 (74 – 90.1)
DS/AD	Down's syndrome patients with AD-related pathology sufficient for the pathological diagnosis of AD	3	45 (12 – 65)
Pick's	Pick's disease	2	63.5 (59, 68)
Total		31	

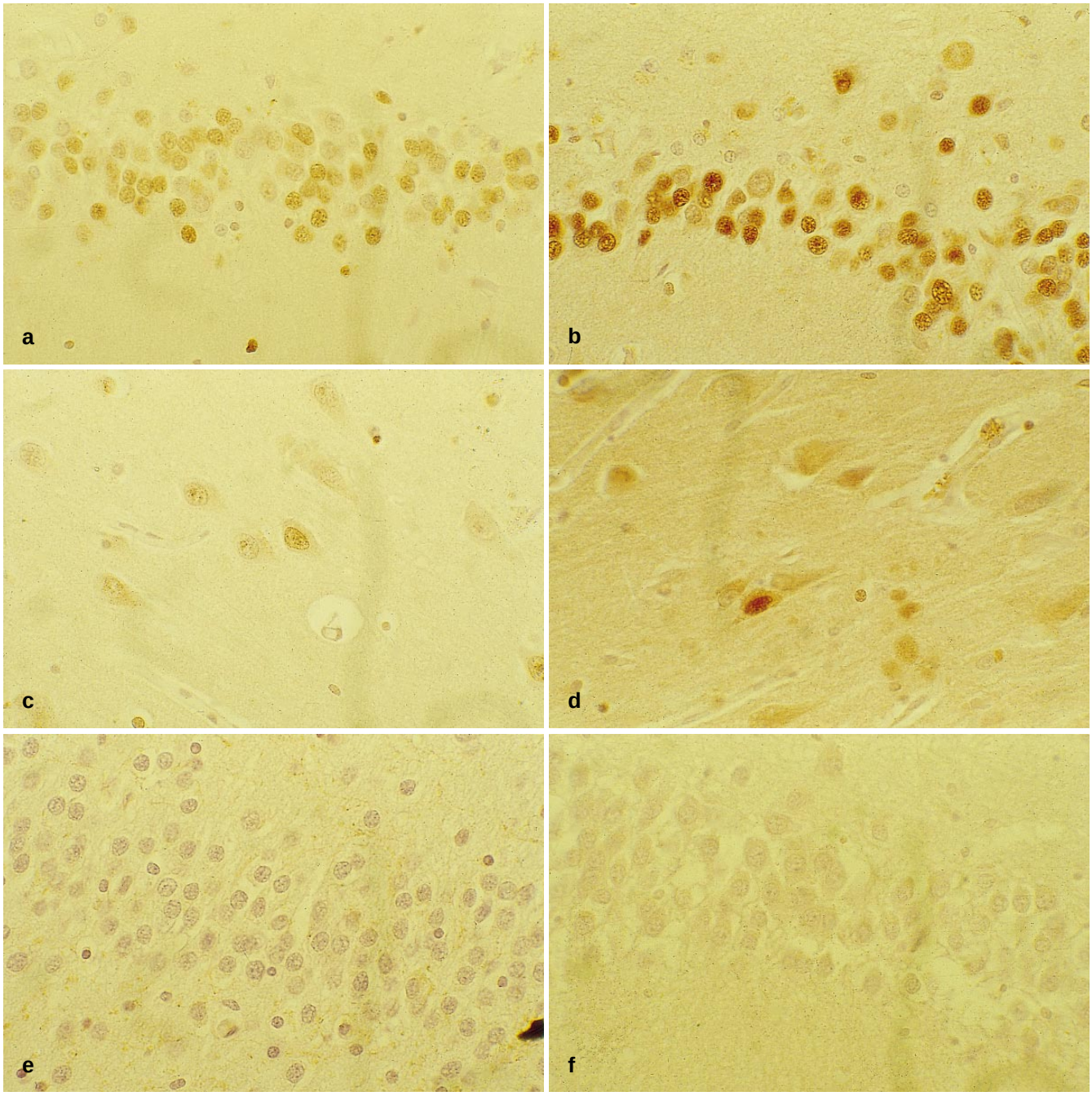

Fig. 2 a–f Cyclin expression in the hippocampus. **a** Cyclin E expressin in the dentate gyrus; **b** cyclin B expression in the dentate gyrus; **c** cyclin E expression in the CA1 region of the hippocampus; **d** cyclin B expression in the CA1 region of the hippocampus; **e** cyclin E labelling in the dentate gyrus after peptide absorption; **f** cyclin B labelling in the dentate gyrus after peptide absorption

the chromogene used for AT8 was 3-amino-9-ethylcarbazole, while cyclin B labelling was visualised with fluorescein (avidin-FITC, Vector Labs). Methanamine silver stain was used to visualise plaque pathology [20] both in hippocampal sections and adjacent neocortical areas.

Quantification of cyclin antibody-labelled nuclei, AT8-positive cells and density of plaques was carried out using the VIDAS 21 image analysis system. Cyclin antibody-labelled nuclei were counted for each patient in five to ten microscopic fields at × 10 magnification in each of the different subfields of the hippocampus. We use the term labelling index (LI) to indicate the mean percent of nuclei labelled in each subfield. A similar LI was calculated for AT8-labelled cells, while the amount of neuritic and diffuse plaques was expressed as the number of plaques per mm^2. The mean LI was calculated from the measurements on all subjects, including those in which the antigen could not be detected.

Positive controls were provided by labelling sections of glial and lymphomatous tumour tissue, fixed in 10% formalin, with all four antibodies (Fig. 1 a–d). To control the specificity of our antibody labelling we performed peptide-absorption assays for cyclins E and B (Fig. 1 e–f). After peptide absorption the immunoreactivity for cyclins E and B in tumour and other sections was abolished.

Statistical analysis was carried out using the Statgraphics software. To analyse the presence of cyclin-immunoreactive cells in

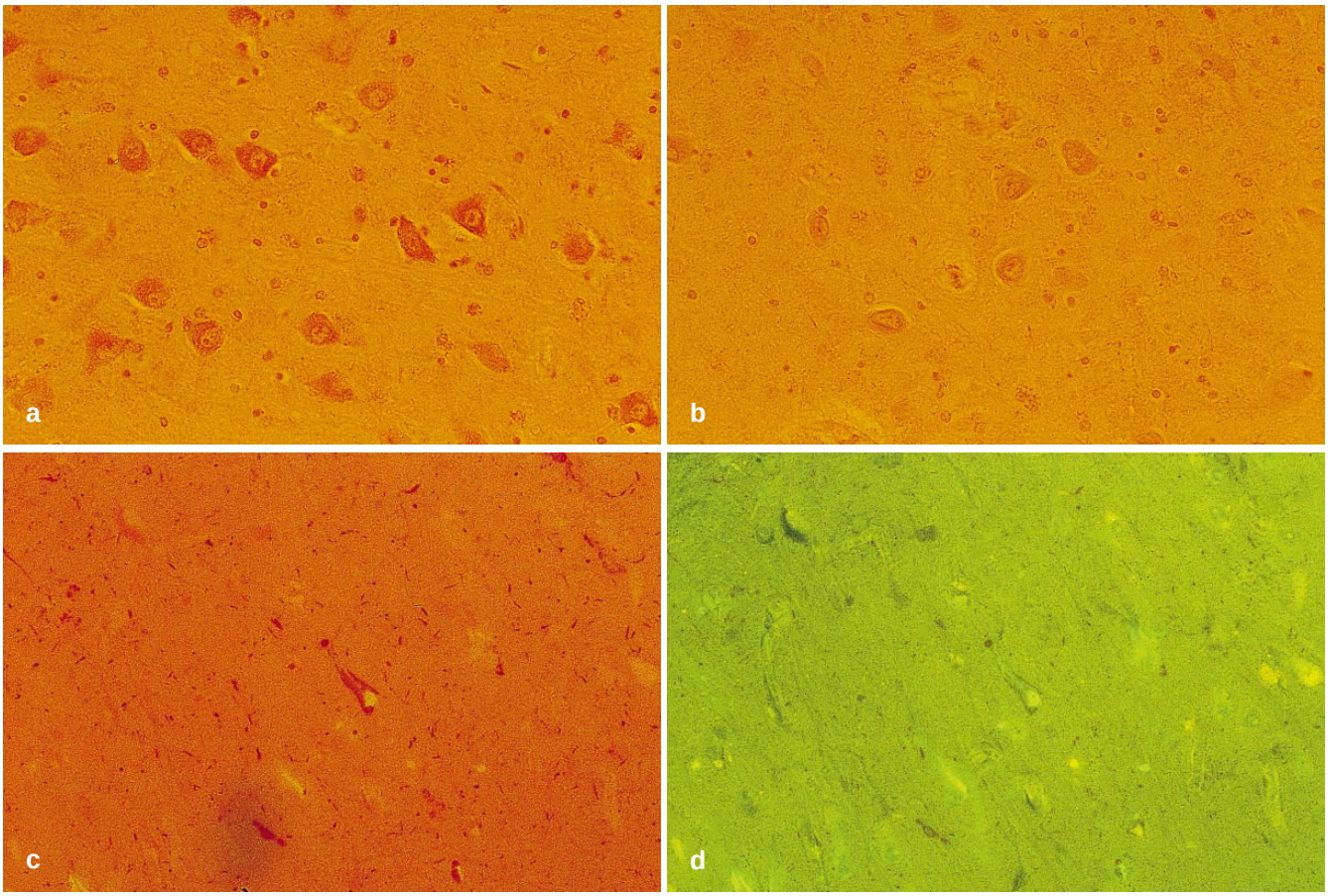

Fig. 3 a–d Cyclin expression in the hippocampus. **a** Cyclin A expression in the CA1 region of the hippocampus; **b** cyclin D expression in the CA1 region of the hippocampus; **c** AT8 expression in hippocampal neurons; **d** cyclin B expression co-localised with AT8 in hippocampal pyramidal neurones

the hippocampus, we subdivided our patients into subgroups according to their pathological diagnosis and the presence or absence of AD-related pathology. The diagnosis of AD was made according to the Tierney A1 criteria [30]. Patients who did not met these criteria for AD but had some AD-related pathology (AT8-positive cells or argyrophilic plaques in their hippocampus) were considered as preAD cases. Patients who did not have any AD-related pathology were considered as controls (Table 1). Patient groups were compared using the one way ANOVA test. Differences between groups were considered to be significant if $P \leq 0.05$. Since large numbers of correlation analyses were performed we applied the Bonferroni correction factor (k) for each group of correlations separately.

Results

Nuclear labelling in hippocampal neurones was detected only for cyclins E and B (Fig. 2). Antibodies to cyclins D and A produced varying degrees of cytoplasmic labelling (Fig. 3 a, b), the significance of which is doubtful and which is not considered further in the present analyses. We also found varying numbers of glial nuclei labelled with all four cyclin antibodies (data not shown); this finding is also not considered further in this study.

Table 2 Number of patients with nuclear cyclin expression in the hippocampus

Patient group	No. of patients	No. of patients with cyclin E expression No. (%)	No. of patients with cyclin B expression No. (%)
C	3	2 (66.6)	0 (0)
DS	2	1 (50)	1 (50)
Pre AD	16	15 (93.7)	13 (81.2)
C/preAD	7	7 (100)	7 (100)
DS/preAD	2	2 (100)	1 (50)
Epil/preAD	2	2 (100)	2 (100)
pre/AD+	5	4 (80)	3 (60)
AD	8	8 (100)	7 (87.5)
AD	5	5 (100)	4 (80)
DS/AD	3	3 (100)	3 (100)
Pick's	2	2 (100)	2 (100)
Total	31	28 (90.3)	23 (74.2)

Labelling with cyclin E

Nuclear cyclin E expression was detected in neurones in all patient groups including controls. In 3 of 31 patients (9.6%) we did not detect cyclin E expression in any of the hippocampal subfields (Table 2). In control subjects the nuclear cyclin E LI was below 10% in all regions of the

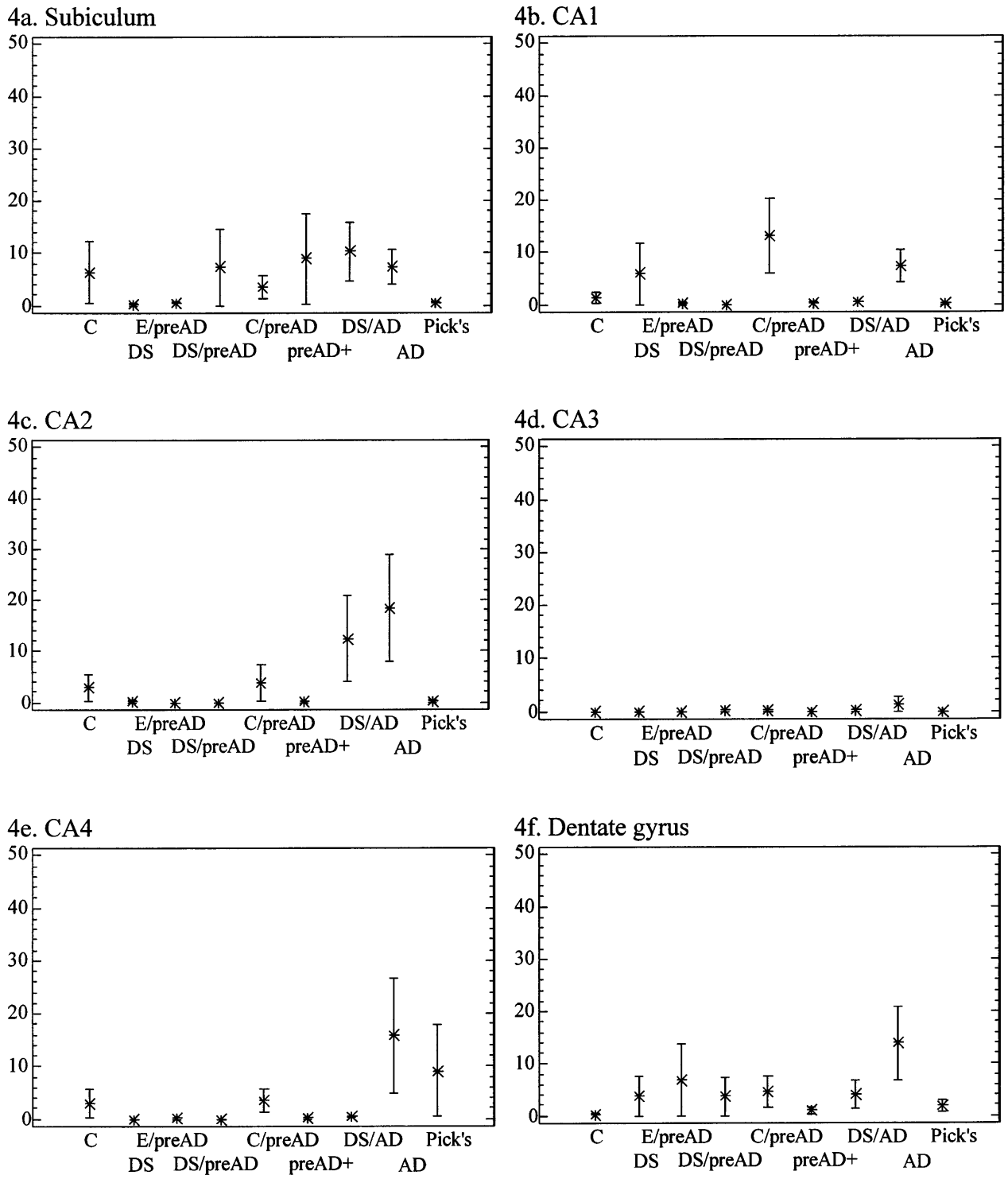

Fig. 4 a–f Cyclin E expression in the subfields of the hippocampus. Distribution of nuclear cyclin E labelling. *x axis* Patient groups in our study. Mean value of the labelling indicies (LI) of all patients of each group is indicated by the *asterisk; bars* indicate the standard error (*AD* Alzheimer's disease, *C* control subjects, *DS* patients with Down's syndrome without AD-related pathology, *E/preAD* epileptic patients with AD-related pathology insufficient to make the diagnosis of AD, *DS/preAD* Down's syndrome patients with some AD-related pathology insufficient to make the diagnosis of AD, *C/preAD* patients with no other than AD-related pathology insufficient for the diagnosis of AD, *PreAD+* cortical Lewy body disease patients with AD-related pathology insufficient for the diagnosis of AD, *DS/AD* Down's syndrome-related AD, *Pick's* Pick's disease patients). *y axis:* LI = the ratio of labelled cells expressed in percent

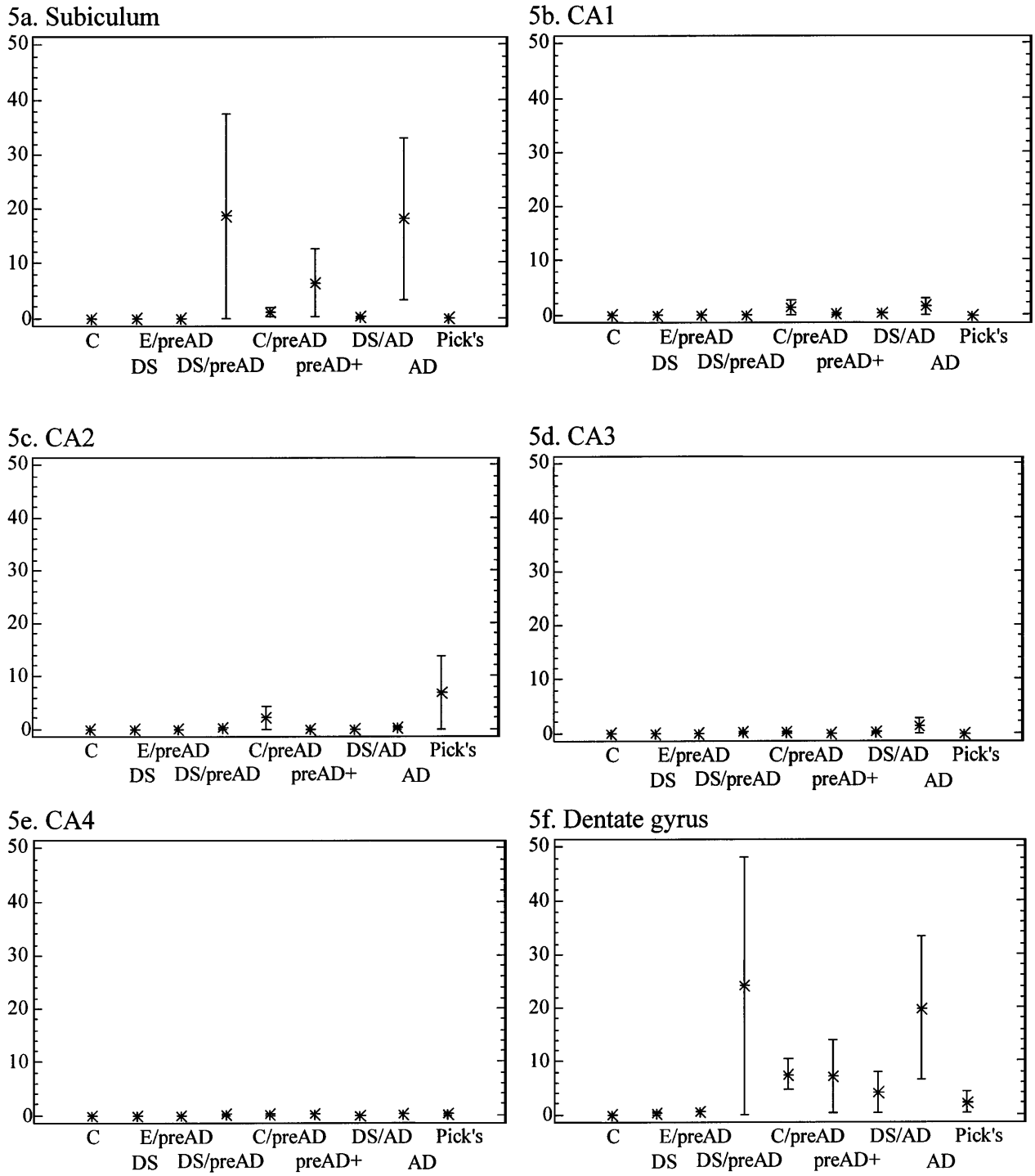

Fig. 5 a–f Cyclin B expression in the different subfields of the hippocampus. Distribution of nuclear cyclin B labelling. *x axis* Patient groups in our study. Mean value of the LI of all patients of each group is indicated by the *asterisk; bars* indicate the standard error. Definition of groups as in Fig. 4. *y axis* LI = the ratio of labelled cells expressed in percent

hippocampus (Fig. 4). In the subiculum, cyclin E was expressed in most patient groups and there were no significant differences between them. In the CA1 field of the hippocampus of the C/preAD, AD and Down's syndrome (DS) groups around 10% of nuclei were labelled with cyclin E antibody and reactions in the other patient groups were negative (for definition of groups see Table 1). Labelling in the CA2 region of C/preAD cases was similar to the control group, while the DS/AD and AD patient groups expressed cyclin E in a larger proportion (10–20%) of nuclei. In CA3 the expression of cyclin E was significantly higher in AD patients than in preAD

cases, while in CA4 the same group showed significantly higher cyclin E expression than the preAD+ cases. The dentate gyrus expression of cyclin E was significantly higher in AD patients than in controls and preAD+ cases.

Labelling with cyclin B1

No cyclin B1 expression could be detected in neurones in the hippocampi of control subjects (Table 2) and DS cases without any AD-related pathology, while preAD and AD cases, both sporadic and DS-related, expressed nuclear cyclin B1 (Fig. 5). In DS cases the cyclin B1 could not be detected in the hippocampus of the DS subjects without AD-related pathology, in the 2nd decade, but was strongly expressed in the subiculum and dentate gyrus in preAD DS subjects and in the dentate gyrus in DS subjects with fully developed AD pathology. In sporadic cases of preAD, cyclin B1 was expressed at low levels in the subiculum, dentate gyrus and CA2 (LI ≤ 10%). The LI doubled as the disease reached the AD stage. Sproadic AD-related pathology in epileptic patients was not associated with cyclin B1 expression. In double-labelled sections cyclin B was invariably co-localised with AT8 in cells that did not have fully developed tangles (Fig. 3 c, d). The exception was the dentate gyrus, where there were relatively few AT8-positive cells but there were high numbers of cyclin B-positive cells. Pick's disease patients also expressed nuclear cyclin B1, but the areas mainly affected in this disease were the CA2 field of the hippocampus and the dentate gyrus (Fig. 5).

Relationship between cells expressing cyclins and plaque or cytoskeletal pathology

There was no statistically significant relationship between cyclin E or B1 and AT8 expression in any of the hippocampal regions examined (Bonferroni correction factor: $k = 36$, requires $P \leq 0.0013$). Cyclin E expression in the dentate gyrus was significantly related to neuritic plaque densities in the neocortex ($P = 0.0003$) (Bonferroni correction factor: $k = 42$, requires $P \leq 0.0011$).

Relationship between cells expressing cyclins and age of the patients

In our patient groups there was no relationship between the expression of cyclins and the age of the patients (Bonferroni correction factor: $k = 12$, $P \leq 0.004$).

The nuclear expression of cyclins was not influenced by the time between death and autopsy in these patients (data not shown).

Discussion

We detected no nuclear labelling with cyclins D and A. However, the presence of immunohistochemically detectable nuclear cyclin E and B was unequivocal. The distribution pattern and the extent of labelling with the two cyclins showed clear differences. The expression of cyclin E showed changes throughout the development of both sporadic and DS-related AD. In DS cases cyclin E expression in the subiculum and dentate gyrus increased with the amount of AD-related pathology, while expression in the CA1 region was highest in DS cases without AD pathology and disappeared as AD-type pathology developed. In the sporadic cases (C/preAD and AD groups) the expression of cyclin E increased in all areas as the AD pathology developed with the exception of CA1, where cyclin E expression decreased with the increase of AD-type pathology. Cyclin B expression, in contrast, was restricted to patients who showed some AD-related pathology and it was present in areas involved in AD pathology, e.g. subiculum, dentate gyrus and CA1. In some patient groups the mean LI was as high as 18% in the subiculum and around 20% in the dentate gyrus (DS/preAD and AD). There was also slight expression of cyclin B in Pick's disease, suggesting that other neurodegenerative diseases besides AD may show perturbations in hippocampal cyclin expression.

The reaction for cyclins E and B1 in neuronal nuclei indicates that these neurones might have re-entered the cell cycle and they are not in the expected G_0 quiescent phase. The absence of nuclear cyclin A and cyclin D1, however points to an inadequate control of cell cycle progression, possibly leading to arrest of the cycle at the different checkpoints in different cells.

The presence of growth factors, or growth factor-like effects, leads cells to re-enter the cell cycle [5]. The further fate of the cell depends upon the expression and activation of the cell cycle regulation machinery [7], some of which is provided by the cyclins [23]. Depending on the expression/activation of cyclins, the cell may progress towards cell division or may be arrested at the different checkpoints of the cell cycle. Previous studies indicate that re-entry into the cell cycle with consequent arrest will lead either to differentiation or to cell death via an apoptotic pathway [7]. The pathway that cells follow, i.e. apoptosis or differentiation, will depend on the phase of the cycle in which cells were arrested and on the presence of growth and other factors [7]. The expression of some cyclins as found in this study indicates that it is possible in vivo for neurones to re-enter the cell cycle. It has been found that amyloid plaques, of the type found in AD patients, contain increased amounts of potentially mitogenic growth factors [2, 6, 18]. Furthermore, AD-type APP has a growth factor-like effect in vitro [1] and the processing of APP during the G_2 phase of the cell cycle leads to the accumulation of amyloidogenic fragments [29]. The phosphorylation pattern of tau in paired helical filaments can be brought about by kinases associated with the cell cycle:

mitogen-activated kinases [3], glycogen synthase kinase 3β and cdk5 which seem to be responsible for tau phosphorylation in AD [23]. Therefore, it is not impossible to envisage the re-entry of neurones into the cell cycle in patients with AD-related pathology. It is interesting that similar conclusions have been reached recently based on independent observations of expression of mitotic protein kinases in neurones in AD [31]. However, the lack of cyclin D1 expression in our material indicates that growth factors needed for the progression of the cycle are not present in adequate amounts to support cell division [16, 28]. The absence of cyclin A supports this view by indicating that DNA replication probably does not occur in hippocampal neurones.

Cyclin E activation is necessary for the progression of the cycle through the G_1/S checkpoint [26, 28]. This transition also requires the expression of cyclin A [26]. This leads us to believe that cells in which we have detected cyclin E but not cyclin A were arrested at the G_1/S transition checkpoint, which raises the question of the fate of these cells. Do they differentiate or do they die via apoptosis? Previous research on apoptosis indicates that cells arrested in G_1 prior to the production of cyclin A have the ability to exit the cycle and differentiate, while for the activation of the apoptotic pathway the expression of cyclin A is necessary [19]. Cells that express cyclin E, with no cyclin A expression may, therefore, still be capable of leaving the cycle and re-acquiring a differentiated phenotype. Expression of cyclin B is a feature of G_2 in cells that have passed beyond the S phase of the cycle. These cells are probably arrested at the G_2/M checkpoint due to the lack of prior DNA replication and/or inactivation of cyclin B, both of which follow from the lack of cyclin A expression [10, 16]. These cells, however, would not be expected to have the capability of differentiating again and would be expected to die via an apoptotic mechanism [5, 13].

The expression of cyclin E, although it is present in elderly controls in a small numbers of neurones, appears to be more prevalent in patients who have some AD-related pathology, reflecting an activation of cycle-related phenomena in an area rich in plaques and tangles. Furthermore, cyclin E expression in the dentate gyrus was significantly related to the neuritic plaque densities in the neocortex. We do not have a clear explanation for this finding. Control patients were never found to show cyclin B expression, in contrast both to AD and to preAD patients in whom high cyclin B labelling was found in areas which are known to be affected severely by AD-related plaque and/or tangle pathology and cell death [32] in AD (CA1, subiculum and dentate gyrus). In these patients in the regions that were less affected by cell loss or AD-type pathology (CA2, CA3 and CA4), cyclin B was almost absent. Furthermore, cyclin B was found in cells that were AT8 positive; however, cells containing fully developed tangles did not seem to have cyclin B-positive nuclei.

Based on these findings it is tempting to suggest that in elderly control patients neurones may be capable of re-entering the cell cycle in areas which lose their synaptic connections as part of an age-related process [32]. However, in normal circumstances the cycle would be arrested at the G_1/S checkpoint, from where, in the absence of cyclin E activation and cyclin A production, cells can return to the quiescent phase. In AD, however, we propose that the regulation of the cell cycle checkpoints is disturbed, allowing these cells to bypass the G_1 checkpoint and prematurely express cyclin B without prior DNA replication. This phenomenon would be expected to lead to destabilisation of microtubules in G_2 arrest, due to cyclin B [5, 25]. The expression and/or activation of cycle-related phenomena in turn would be expected to activate proline-directed kinases [23] that together with the already destabilised cytoskeleton, could lead to paired helical filament formation and neurofibrillary tangles. Indeed, there are indications that the activation of MAP kinases, which may be able to convert tau into paired helical filament-like tau, is an earlier event than the appearance of neurofibrillary pathology [3]. However, with the formation of tangles the metabolism of the cells may be disturbed to such a degree that the nuclear cyclins disappear. We, therefore, propose that cell cycle disturbances in AD may be early pathogenetic events that appear before AD-type pathology is fully developed.

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