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Predisposition to accelerated Alzheimer-related changes in the brains of human immunodeficiency virus negative opiate abusers

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Cognitive impairment is a recognized effect of drug misuse, including the use of opiates. The pathological basis for this is unknown but the temporal and frontal cortices have been implicated. We have shown previously that deposits of hyperphosphorylated tau in drug user brains exceed those seen in age-matched controls. The present quantitative study of hyperphosphorylated tau and beta amyloid in drug user brains allows comparison with the related pathology in Alzheimer's disease. Brains were obtained from the Edinburgh Medical Research Council Brain Banks, comprising 39 human immunodeficiency virus negative drug users, five subjects with Alzheimer's disease and 37 age-matched, cognitively normal controls, all legally and ethically approved for research. Hyperphosphorylated tau positive (AT8, AT100) neuropil threads were significantly increased in the frontal and temporal cortex, and in the locus coeruleus, of drug users aged >30 years (all $P=0.04$). Under the age of 30 years, drug users showed a similar increase in neuropil threads compared with controls, but this reached significance only in the frontal cortex ($P=0.03$). Immunopositivity for both three- and four-repeat tau was present in drug user brains. There was a direct relationship between the numbers of neuropil threads and of neurofibrillary tangles: neurofibrillary tangles were sparse in brains that had neuropil thread counts below 200 cm^2. Hyperphosphorylated tau positive neuropil threads increased at a faster rate in drug users than in controls and the levels of the phosphorylating enzyme, GSK-3, was raised in drug user brains. Beta amyloid (AB4, AB42 and 4G8) was raised in drug user brains (mainly as shadow plaques) but not significantly different from controls and there was no correlation between high beta amyloid and hyperphosphorylated tau in individual cases. Hyperphosphorylated tau levels correlated significantly ($P=0.038$) with microglial activation in drug users but not in controls. The levels of hyperphosphorylated tau in drug users fell far short of those seen in Alzheimer's disease but overlapped with those in elderly controls. We conclude that drug users show early Alzheimer's disease-related brain pathology that may be the basis for cognitive impairment and that neuroinflammation is an early accompanying feature. This provides an opportunity to study the pathogenesis of tau pathology in the human brain.

Keywords: tau protein; amyloid; drug abuse; opiates

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Introduction

Drug misuse represents a serious financial and social burden in many countries (General Register Office for Scotland, 2005; Office of National Statistics, 2006). In Scotland an estimated 1.8% of 15–54 year-olds are addicted to drugs (Hay *et al.*, 2005) and there has been a significant increase in mortality over the years (Laskus *et al.*, 2005). Drug-related deaths represent almost 1% of all deaths in Scotland, and in 14% of the deaths in the age range 25–45, opiates are usually implicated. Intravenous drug users are also at risk of contracting a variety of blood-borne viral infections, including HIV and hepatitis B and C. Drug abuse and psychiatric disorders, particularly bipolar disorder and major depression, co-exist in a significant proportion of cases (Miller *et al.*, 1989; Maremmani *et al.*, 2000, 2006; Lammertink *et al.*, 2001).

Despite this background, relatively little is known of the long-term impact of drug use on the brain. Although there are difficulties in determining whether cognitive decline is present in drug users, such an association has been established (Robbins *et al.*, 2008). Studies of addicts who abstained from drug intake for 24 h prior to testing showed impairment of psychomotor speed, working memory and of decision making (Ornstein *et al.*, 2000; Ersche *et al.*, 2006). Cognitive impairment has been found to persist for years even in abstinent opiate drug users (Ersche *et al.*, 2006).

At the pathological level, studies to date suggest that there are no brain lesions that can be regarded as specific for drug abuse (Buttner *et al.*, 2000). Opiate-mediated depression of respiration can lead to hypoxic brain injury of varying severity in heroin and methadone users, ranging from cellular and subcellular damage through focal tissue injury in susceptible brain regions to global hypoxia and sudden death. Imaging studies suggest that chronic heroin abusers have cerebral atrophy and that vascular events are not uncommon (Wolf and Mikhael, 1979; Cala and Mastaglia, 1980). Neuropathological examination has confirmed that vascular changes are more common in drug users and these include infarcts, hypoxic injury, and acute and chronic breakdown of the blood–brain barrier (Buttner *et al.*, 2006). Focal accumulation of amyloid precursor protein in the white matter of drug user brains is likely to be the result of hypoxic injury to axons (Buttner *et al.*, 2006).

At the cellular level we have previously reported an increase in the levels of hyperphosphorylated tau in the brains of HIV negative drug users (Ramage *et al.*, 2005) compared with age-matched controls. This insoluble derivative of tau protein is found in abundance, along with beta amyloid, in the brain in Alzheimer's disease and to a lesser extent in the ageing brain. While it is known that very low levels of insoluble tau are observed in otherwise normal young brains (Braak and Braak, 1997*b*), the implications of significantly elevated levels in drug users are currently unclear. Here we extend the investigation of tau metabolism in the brains of opiate abusers and compare this with cases of Alzheimer's disease in terms of hyperphosphorylated tau and beta amyloid distribution and load, and investigate factors that might contribute to hyperphosphorylated tau deposition. These include neuroinflammation in the form of microglial activation, since this has been linked to the early stages of Alzheimer's disease pathogenesis (McGeer *et al.*, 2006).

Materials and methods

The brains of 81 subjects have been examined, including 39 HIV negative intravenous drug users, five subjects with Alzheimer's disease and 37 control cases, age-matched with the former. HIV negative drug users were subdivided into two groups: <30 and >30 years of age. The control cases were subdivided into three groups based on age: <30, >30 and >60 years (Table 1). The mean age of the control group under 30 years was 23 years and the corresponding drug user group was 23.5 years. The mean age of the >30 years control group was 40 years and the corresponding drug user group 39 years. The elderly control group over 60 had a mean age of 75 years and the comparator Alzheimer's disease group had a mean age of 79 years.

All cases were obtained from the Medical Research Council Edinburgh Brain Banks, and authenticated and ethically approved for research studies. Table 1 shows the brain areas examined in these age ranked groups, with mean and median ages for each group.

The control cases were selected on the basis of having no personal or family history of drug abuse, no history of cognitive impairment or other brain disorder, and no obvious neuropathological abnormality at autopsy. Although the drug users had not been regularly assessed for cognitive status, none had a history of overtly impaired cognitive function although depression was common in this group (32%), with a history of attempted suicide in 5%, and 21% had had periods of psychosis. Forty-two percent of drug abusers in this

Table 1 Study groups

	Control (<30) *n* = 14	Intravenous drug abusers (<30) *n* = 21	Control (>30) *n* = 16	Intravenous drug abusers (>30) *n* = 18	Elderly controls (>60) *n* = 7	Alzheimer's disease *n* = 5
Hippocampus[a]	13	21	13	18	7	5
Frontal lobe	12	21	16	18	7	0
Pons (locus coeruleus)	6	12	11	13	6	0
Occipital lobe	4	7	12	4	6	0
Thalamus	6	10	9	6	6	0
Cerebellum	6	10	9	6	6	0
Mean age	23	23.5	40	39	75	79
Median age	22.5	24	41	37	74.5	79

a Sections of hippocampus contained cortex in addition to hippocampus.

study had no documented mental illness. The cause of death in the control group was usually a sudden cardiac event or road traffic accident not involving head injury, and the drug users most commonly died following accidental drug overdose, although several cases had committed suicide. The drug users came predominantly from low socio-economic backgrounds and had mixed patterns of abuse. All were opiate abusers (heroin, morphine and methadone), but supplemented this with a range of other substances including cannabis, di-hydrocodeine, diazepam, amphetamines and nicotine.

Immunohistochemistry

Immunohistochemistry was performed on formalin fixed, paraffin embedded sections of 5 μm thickness, using a tyramide signal amplification or avidin binding complex method, as described previously (Ramage *et al*., 2005). Table 2 lists the antibodies and experimental conditions, including pre-treatments. Tau antibodies AT8 and tau100, and antibodies directed to AB42 and α-synuclein, were used in sections from all available areas in every case listed in Table 1. Antibodies against three- and four-repeat tau were used on sequential sections of cases with high levels of AT8 or AT100 positivity. Antibodies to beta amyloid AB4 and 4G8, as well as major histocompatibility complex class II, were used only on sections from the hippocampus. Blood–brain barrier integrity was studied, in a subset of cases, by comparing the ratio of endothelial cells stained for the endothelial cell marker Von Willebrand Factor with staining for the blood–brain barrier tight junction protein ZO-1. For each case studied, consecutive sections were stained with either ZO-1 or Von Willebrand. Large distinctive vessels present in the tissue section were used to confirm that sections were consecutive. The total number of vessels stained positive with each antibody were counted and a ratio of Von Willebrand to ZO-1 was calculated. There is a degree of inaccuracy in the process as small blood vessels may not be present in both sections even if they are consecutive. However, on average the technique gives a good approximation of the percentage of tight junction staining in tissue sections.

Quantitation

Sections stained for AT8 and AT100 were analysed manually for the presence and total number per section of neuropil threads and neurofibrillary tangles. The total area of each section was then calculated allowing the number of neurofibrillary tangles and neuropil threads per cm^2 to be determined. Sections stained for three- and four-repeat tau were assessed subjectively and independently by F.W.C. and I.C.A. In sections stained for AB42, the total number of positive plaques was manually counted in each section and converted to the number of positive plaques per cm^2. Sections stained for AB4 and 4G8 were assessed subjectively by J.E.B. No test sections displayed positivity for α-synuclein, so no quantitation was required. Major histocompatibility complex class II stained sections were quantified using a computerized image analysis system, Image Pro Plus (Media Cybernetics) because the very numerous and irregular outlines of positive cells precluded accurate manual counts.

Western blot

Western blot for GSK-3 α/β (Abcam) was performed using crude extract from the hippocampus of seven control cases and seven drug users, and from the frontal lobe of six controls and six drug users. ECL-Plus (GE Life Sciences) was used as a visualizing agent. All results were normalized to expression of beta actin protein to

Table 2 Antibodies for immunohistochemistry

Primary antibody	Concentration	Secondary antibody	Concentration	Pretreatment
Amyloid 4G8 (Signet Labs)	1/100	Biotinylated rabbit anti-mouse (DAKO)	1/200	95% formic acid
Beta amyloid 4 (Dako)	1/100	Biotinylated rabbit anti-mouse (DAKO)	1/200	95% formic acid
Beta amyloid 42 (Abcam)	1/100	Biotinylated rabbit anti-mouse (DAKO)	1/200	95% formic acid
MHC class II (DAKO)	1/100	Biotinylated rabbit anti-mouse (DAKO)	1/200	Microwaved in 0.01 M citric acid pH 6.0
Three-repeat tau (Upstate)	1/100	Biotinylated rabbit anti-mouse (DAKO)	1/200	Microwaved in 0.01 M citric acid pH 6.0 followed by formic acid 95%
Four-repeat tau (Upstate)	1/100	Biotinylated rabbit anti-mouse (DAKO)	1/200	Microwaved in 0.01 M citric acid pH 6.0
Tau AT100 (Autogen Bioclear)	1/100	Biotinylated rabbit anti-mouse (DAKO)	1/200	Microwaved in 0.01 M citric acid pH 6.0
Tau AT8 (Autogen Bioclear)	1/100	Biotinylated rabbit anti-mouse (DAKO)	1/200	Pressure cooked in 0.01 M citric acid pH 6.0
Von Willebrand Factor (DAKO)	1/100	Biotinylated swine anti-rabbit (DAKO)	1/200	Pressure cooked in 0.01 M citric acid pH 6.0
ZO-1 (Zymed)	1/100 (overnight at 4°C)	Biotinylated swine anti-rabbit (DAKO)	1/200	Trypsin 37°C 100 mg/100 ml TBS pH 7.8
α-synuclein	1/750	Biotinylated rabbit anti-mouse (DAKO)	1/200	Microwaved in 0.01 M citric acid pH 6.0

MHC = major histocompatibility complex.

allow comparison between cases and to account for potential differences in degradation between samples. Images were analysed and quantified using ImageQuant TL (GE Healthcare).

Western blots for soluble tau and insoluble hyperphosphorylated tau were performed using AT8 antibody (Autogen Bioclear). Separation of soluble tau and insoluble hyperphosphorylated tau was carried out in order to concentrate the amount of hyperphosphorylated tau in samples. Crude protein extracts were created from frozen unfixed tissue. Extract was centrifuged at 100 000 *g* for 60 min at 4°C. Supernatant containing soluble tau was removed and separated from the pellet that was re-suspended in buffer containing 10 mM Tris, 1 mM ethylene glycol tetraacetic acid and 10% sucrose. The solution was centrifuged at 15 000 *g* for 20 min at 4°C. Supernatant was removed and 1% sarkosyl added, the solution was mixed for 1 h and then centrifuged at 100 000 *g* for 30 min at 4°C. The supernatant was discarded and the pellet containing crude hyperphosphorylated tau was resuspended in 50 mM Tris.

ApoE genotyping

ApoE genotyping for the three common alleles E2, E3 and E4 was carried out using enzyme restriction digest of a 222 bp polymerase chain reaction product amplified from the *ApoE* gene as described previously (Becher *et al.*, 2008).

Statistics

Statistical analysis was performed to determine differences between drug users and their age-matched control group. The Mann–Whitney U-test was used for inter-group comparisons, and correlations between hyperphosphorylated tau load and other factors, including age and level of neuroinflammation were determined using Pearson's Correlation.

Results

Hyperphosphorylated tau

Hyperphosphorylated tau positive neuropil threads (Fig. 1) were quantified in five key areas of the brain. The results are shown in Table 3 as median values for each area in control and drug user brains, confirming the increase in hyperphosphorylated tau burden in drug user brains. The cerebellum is not included since it displayed virtually no hyperphosphorylated tau in any case below the age of 60 years.

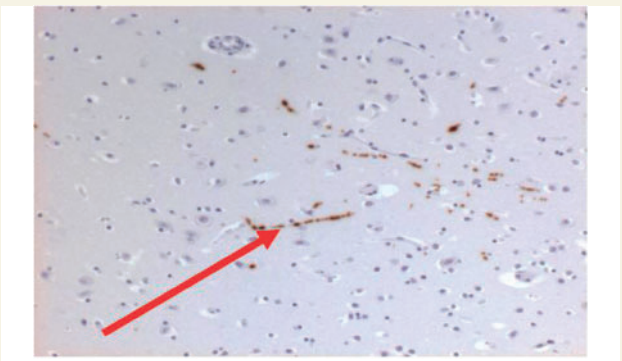

Figure 1 Hyperphosphorylated tau expression in neuropil threads of a drug abuser.

In the hippocampus the increased number of neuropil threads in drug users compared with controls (Fig. 2) reached statistical significance for the >30 years age group ($P = 0.04$) but failed to reach significance in the <30 years group. Similar increases were noted in the frontal lobe (Fig. 3) with drug users aged >30 years and under having significantly greater levels of hyperphosphorylated tau positive neuropil threads ($P = 0.04$ for >30 years and $P = 0.03$ for <30 years). In the locus coeruleus of the pons, a significant increase was noted in the >30 years drug abusers ($P = 0.04$) but not in the <30 years group ($P = 0.2$) (Fig. 4). In the occipital lobe, the trend for higher values in drug users was also present but too few cases were available to perform meaningful statistical analysis. In the thalamus no significant difference was observed between drug users and controls ($P = 0.44$ for >30 years and $P = 0.33$ for <30 years). The range of hyperphosphorylated tau values seen in drug users aged 30–60 years overlapped with that seen in elderly controls (Figs. 2–4) but not with those found in cases of Alzheimer's disease (2×10^4 to 2×10^6 neurites per cm^2) (data not shown).

Figure 5 shows hyperphosphorylated tau levels in drug users and controls plotted against age, and reveals that the rate of accumulation of hyperphosphorylated tau is greater in the drug user group than for controls. The values for Alzheimer's disease cases have also been included in Fig. 5, from which inferences may be drawn about the onset of this disorder in terms of hyperphosphorylated tau deposition (see 'Discussion' section).

Hyperphosphorylated tau positive neurofibrillary tangles were counted in each of the six brain regions. None of the controls below the age of 60 years had >1 neurofibrillary tangle per cm^2 but after 60 years nearly all had >1 neurofibrillary tangle per cm^2 (Fig. 6). Two drug users (aged 24 and 56 years) had considerably more neurofibrillary tangles (Fig. 6). In the hippocampus the number of neurofibrillary tangles exceeded 1 per cm^2 only in brains that displayed more than 200 (or $10^{2.3}$) neuropil threads per cm^2 (Fig. 7) and this applied to both drug users and controls. A significant correlation was noted between the number of neurofibrillary tangles and that of neuropil threads in the hippocampus ($P = 0.017$, $r = 0.705$ for controls and $P = 0.005$, $r = 0.641$ for drug abusers). This correlation was also found in the frontal lobe of drug users ($P = 0.002$, $r = 0.762$) but although there was a

Table 3 Median values for neurites in multiple brain regions

Brain area	Controls (<30)	Drug abusers (<30)	Controls (>30)	Drug abusers (>30)
Hippocampus	0.41	1.57	14.38	64.27
Frontal lobe	1.28	8.81	6.23	19.98
Occipital lobe	0.00	3.33	0.00	7.26
Thalamus	0.50	2.16	5.50	1.17
Locus coeruleus	5.50	2.00	7.50	36.75

All values are median number of neurites per square centimetre.

similar trend in control frontal lobes, too few control cases had tangles present to perform meaningful statistical analysis. On the basis of these findings we postulate a threshold value of neuropil threads for the development of neurofibrillary tangles (Fig. 7).

Expression of phosphorylating enzymes

Western blot analysis of GSK-3 expression in the hippocampus and frontal lobe of drug abusers demonstrated a significantly elevated level of GSK-3 expression in the hippocampus ($P = 0.03$) but not frontal lobe ($P = 0.09$) of drug users compared to age-matched controls (Fig. 8). A trend was noted for cases that displayed higher hyperphosphorylated tau levels to also have higher levels of GSK-3. However, too few cases were studied for meaningful statistical analysis to be undertaken.

Expression of Cyclin Dependant Kinase 5 (CDK-5) was also examined and showed a similar pattern to that observed with Glycogen Synthase Kinase 3 (GSK-3), with a significantly elevated levels found in the hippocampus ($P = 0.045$) but no difference observed in the frontal lobe ($P = 0.33$) (Fig. 9).

Molecular analysis of tau in drug abusers

Analysis by western blot of soluble tau and insoluble hyperphosphorylated tau confirmed that all five cases of Alzheimer's disease displayed the expected triplet repeat pattern for hyperphosphorylated tau (Fig. 10). Frozen tissue was available only in a limited number of drug users, unfortunately all with neuropil thread counts of $<10^2$ per cm^2, and none with sufficient hyperphosphorylated tau for this type of analysis.

We sought to further characterize hyperphosphorylated tau in drug users using a pair of antibodies that recognize the three- and four-repeat versions of tau. In drug users with high hyperphosphorylated tau burdens we observed expression of both three- and four-repeat tau, similar to findings in Alzheimer's disease.

Beta amyloid expression

Staining for AB4 showed subpial deposits and/or plaques in the temporal cortex in 23% of drug users compared with 14% of age-matched controls (Fig. 11). A different pattern of beta amyloid deposits was revealed with the antibody 4G8, including focal to widespread staining of neuronal cell bodies in all cases but more prominent in drug users, as well as cortical shadow plaques in 18% of drug users and 10% of matched controls (Fig. 12). These differences were not significant. AB42 was deposited in the entorhinal cortex as occasional compact cortical plaques and perivascular deposits in 22% of drug users and in 17% of controls ($P = 0.6$). In the frontal lobe, 13% of control subjects and 32% of drug abusers had >1 positive AB42 structure ($P = 0.08$). In contrast, 33% of controls over the age of 60 had AB42 positivity in both

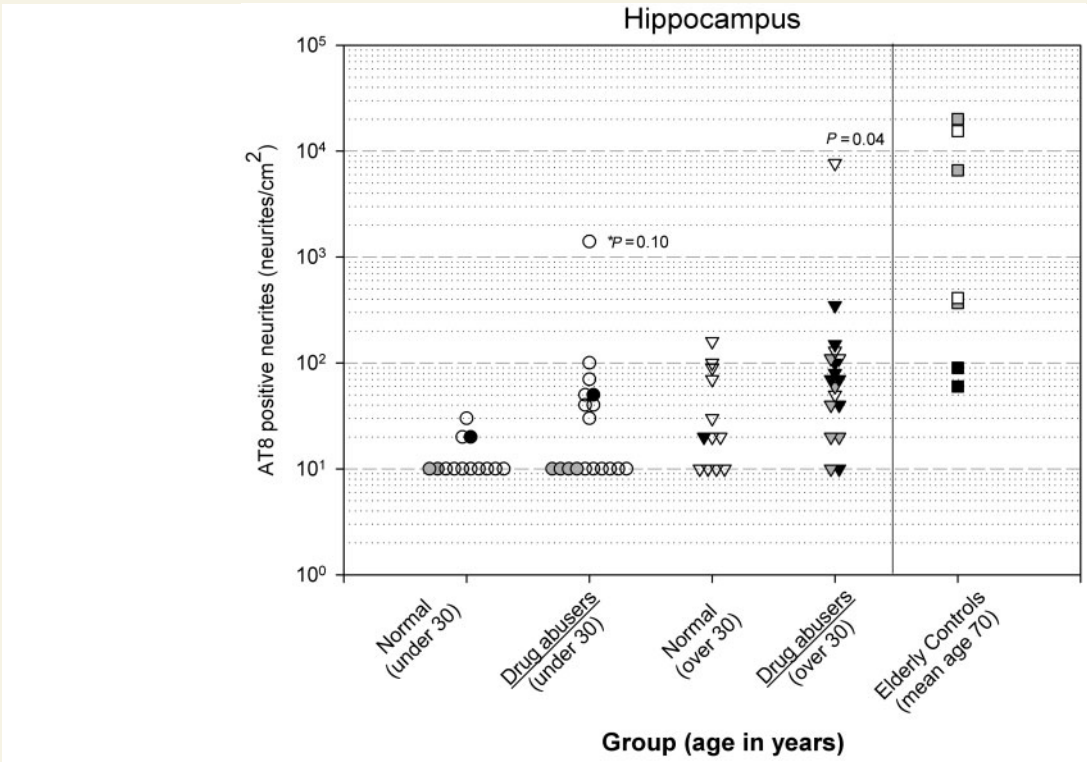

Figure 2 Comparison of hyperphosphorylated tau positive neuropil threads in the temporal cortex of two age groups of drug users and control subjects. Data from elderly control subjects are presented for comparative purposes. A significant difference was detected between drug users and controls over the age of 30 years ($P = 0.04$). (Note: logarithmic scale used.). Black symbol denotes cases positive for BA42 in the hippocampus. Grey symbol denotes cases positive for BA42 in other brain regions examined.

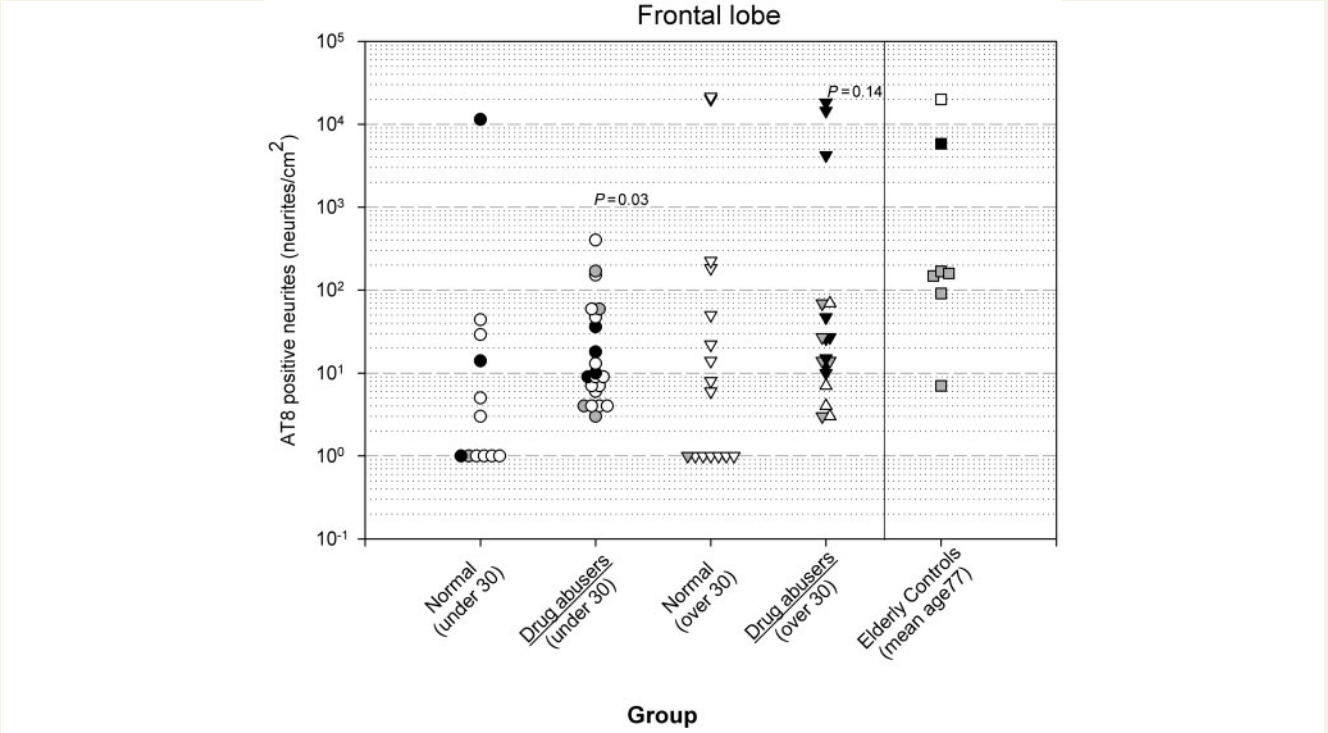

Figure 3 Comparison of hyperphosphorylated tau positive neuropil threads in the frontal cortex of two age groups of drug users and control subjects. Data from elderly control subjects are presented for comparative purposes. A significant difference was detected between drug users and controls under the age of 30 years ($P = 0.03$) and over the age of 30 years ($P = 0.04$). (Note: logarithmic scale used.). Black symbol denotes cases positive for BA42 in the frontal lobe. Grey symbols denote cases positive for BA42 in other brain regions examined. White symbol denotes cases with no BA42 positivity.

the hippocampus and frontal lobe. All cases of Alzheimer's disease displayed extensive AB42 positivity. Under the age of 60 years, there was no evidence of a correlation between AB42 deposition and increasing age, in drug users or in controls, unlike our finding for hyperphosphorylated tau accumulation in drug users.

Beta amyloid and hyperphosphorylated tau

No correlation was found between the presence of beta amyloid deposits and the hyperphosphorylated tau load in neuropil threads, either in control subjects or in drug users (hippocampus: controls $P = 0.82$, drug users $P = 0.77$; frontal lobe: controls $P = 0.80$, drug users $P = 0.78$).

Blood–brain barrier integrity

Blood–brain barrier structural integrity was determined by comparing expression of the tight junction protein ZO-1 with endothelial cell marker expression (Von Willebrand Factor). Sections that showed blood vessels with matched ZO-1 and Von Willebrand Factor staining were defined as having high levels of blood–brain barrier structural integrity (Fig. 13). Sections with blood vessels lacking ZO-1 expression but still showing Von Willebrand Factor expression were defined as having lower blood–brain barrier structural integrity (Fig. 13). Figure 14 shows significantly reduced blood–brain barrier integrity was observed in drug users versus controls ($P = 0.05$).

Inflammation

No significant difference in the expression of major histocompatibility complex class II on microglial cells was noted between drug users and controls aged under or over 30 years. However there was a significant correlation between the degree of microglial activation, as revealed by major histocompatibility complex class II positivity, and the extent of hyperphosphorylated tau deposition in drug users but not in controls (Fig. 15).

ApoE genotype

ApoE genotype revealed a distribution of the three main alleles (E2, E3 and E4) in the frequency 10% E2, 70% E3 and 20% E4 that differs from previously published data on the Scottish adult population, which shows 8% E2, 77% E3 and 15% E4 (Cumming and Robertson, 1984). No significant correlation was noted between E4 and the presence of hyperphosphorylated tau.

Discussion

The process of tau accumulation in normal and diseased brains is still poorly understood. Most human studies, by necessity, focus

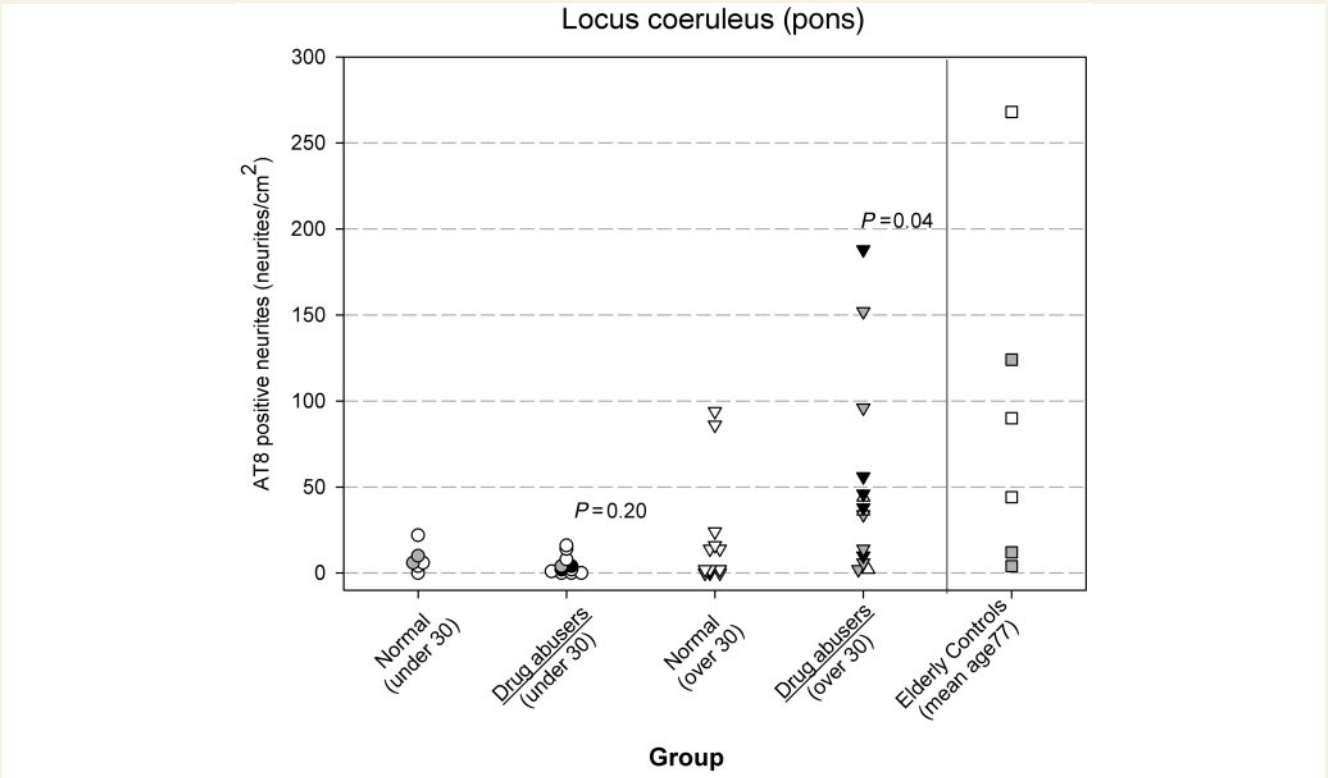

Figure 4 Comparison of hyperphosphorylated tau positive neuropil threads in the locus coeruleus of two age groups of drug users and control subjects. Data from elderly control subjects are presented for comparative purposes. A significant difference was detected between drug users and controls over the age of 30 years ($P = 0.04$). Black symbol denotes cases positive for BA42 in the frontal lobe. Grey symbols denote cases positive for BA42 in other brain regions examined. White symbol denotes cases with no BA42 positivity., Edinburgh, E48 9AG, UK. Black symbol denotes cases positive for BA42 in the frontal lobe. Grey symbols denote cases positive for BA42 in other brain regions examined. White symbol denotes cases with no BA42 positivity.

on end-stage disease with limited opportunities to study the pathology of the early phases of dementias. This study confirms and extends our earlier findings of an increase in hyperphosphorylated tau deposition in the brains of drug users (Ramage *et al*., 2005), relating this both to microglial activation and to breakdown of the blood–brain barrier, but finding no correlation with deposition of beta amyloid. Because the subjects are much younger and the load of insoluble proteins much lighter than those in Alzheimer's disease, investigations of this kind afford an insight into the early stages of tau and amyloid pathology.

Study cohorts of drug users are notoriously diverse because of polydrug abuse, as well as variations in drug intake and abstention, and in adherence to clinical follow-up. Our cohort was selected for opiate misuse and originate from a relatively stable geographic setting, but the wider variation in data points and larger standard deviations among the drug users as compared with controls, probably reflect the heterogeneity among drug users. Despite these difficulties, significant differences are evident in terms of tau deposition between drug users and controls. We recognize that these results reflect what is present in drug users who have died prematurely of drug overdose and who may have had more chaotic drug habits than those who survive. It is unclear whether raised levels of hyperphosphorylated tau are linked to psychiatric disorder in our cohort and we are not able to comment on any link with subtle cognitive impairment. Prosser *et al.* (2006) have shown that patients on methadone maintenance therapy exhibit similar degrees of cognitive dysfunction to those of former heroin abusers who are in a prolonged period of abstinence. Similar findings have been reported by Ersche *et al.* (2006). These studies suggest that damage caused to the brain by long-term drug use may not be reversible. Other reported changes at the cellular level associated with drug use include decreased neuronal densities in the basal ganglia of heroin addicts (Pearson *et al.*, 1976), which correlates with reports of hypoxia and ischaemia in the same region (Andersen and Skullerud, 1999). Morphine induced apoptosis has been demonstrated in both neurons and microglia (Hu *et al.*, 2002). Opiate-induced damage to the neuronal cytoskeleton includes decreased levels of α-tubulin messenger RNA and protein in rats exposed to chronic morphine treatment (Marie-Claire *et al.*, 2004), and aberrant neurofilament phosphorylation in the prefrontal cortex in opioid abusers (Ferrer-Alcon *et al.*, 2000). Treatment of mice with 3,4-methylenedioxymethamphetamine (MDMA) has been reported to impair learning and memory while enhancing tau phosphorylation in the hippocampus (Busceti *et al.*, 2008).

In Alzheimer's disease, hyperphosphorylated tau is found together with beta amyloid and appears to be required for the clinical expression of the disease (McKee *et al.*, 1991; Braak and

Figure 5 Accumulation of hyperphosphorylated tau positive neuropil threads in the hippocampus in both control subjects and drug abusers correlated with age. The data show a significant correlation with age in both controls ($P = 0.002$) and drug abusers ($P = 0.004$). Data are also presented from Alzheimer's disease cases. The rate of accumulation for drug users is indicated by a black line and in controls by a grey line. Corresponding neuropil thread values for Alzheimer's disease cases are included, with a dashed line indicating a hypothetical rate of hyperphosphorylated tau positive neuropil threads for Alzheimer's disease (see 'Discussion' section). Based on the lowest level of tau burden observed in Alzheimer's disease cases with clinical dementia and the highest level observed in control subjects with no history of dementia we have suggested a window of clinical onset for dementia in terms of hyperphosphorylated tau neuropil thread accumulation. (Note: logarithmic scale used.). Significant correlation between age and Tau for both drug abusers ($P = 0.004$, $r = 0.43$) and control subjects ($P = 0.002$, $r = 0.545$).

Braak, 1997*a*). In other tauopathies such as frontotemporal dementia with Parkinsonism linked to chromosome 17, corticobasal degeneration, Pick's disease and progressive supranuclear palsy, hyperphosphorylated tau is sufficient for the process of neurodegeneration to occur (Pollock *et al.*, 1986; Braak and Braak, 1997*b*; Hutton *et al.*, 1998). Although it is currently debated as to whether accumulating insoluble proteins such as hyperphosphorylated tau and beta amyloid are harmful, mere bystander phenomena or are actually neuroprotective (Bretteville and Planel, 2008); they are certainly found at high levels in Alzheimer's disease, and linked quantitatively to dementia (McKee *et al.*, 1991; Braak and Braak, 1997*a*). Although beta amyloid plaques and neurofibrillary tangles are found, often in quantity, in the brains of elderly non-demented individuals, these are usually regarded as representing preclinical stages of Alzheimer's disease (Neuropathology Group of the Medical Research Council Cognitive Function and Ageing Study, 2001). Ridding the brain of these two proteins is the focus of current therapeutic approaches (Holmes *et al.*, 2008).

In this study we have demonstrated, and quantified, the increased deposition of hyperphosphorylated tau in multiple brain regions of opiate drug abusers. Antibodies to three- and four-repeat tau have been validated in previous studies of human dementias (de Silva *et al.*, 2006). Both three- and four-repeat hyperphosphorylated tau were present in drug user brains, showing some of the characteristics of hyperphosphorylated tau deposited in Alzheimer's disease or frontotemporal dementia with Parkinsonism linked to chromosome 17, and in this respect differing from hyperphosphorylated tau found in Pick's disease (characteristically three-repeat). Beta amyloid deposits showed a wider degree of variation between brains so the increase observed in drug user brains compared with controls did not reach significance. More importantly, brains containing high levels of hyperphosphorylated tau did not necessarily show beta amyloid deposits, suggesting that accumulation of these two proteins may be independent of each other, at least initially.

It is recognized that hyperphosphorylated tau starts to accumulate in otherwise apparently normal brains from early adulthood (Braak and Braak, 1997a), confirmed in the present study. Here we show that drug users accumulate hyperphosphorylated tau at a faster rate compared to age-matched controls. It has been claimed that neuropil thread counts correlate with dementia (McKee *et al.*, 1991). However, no such correlation has been found in other similar studies (Masliah *et al.*, 1992). We have also shown that there appears to be a critical threshold for neuropil thread

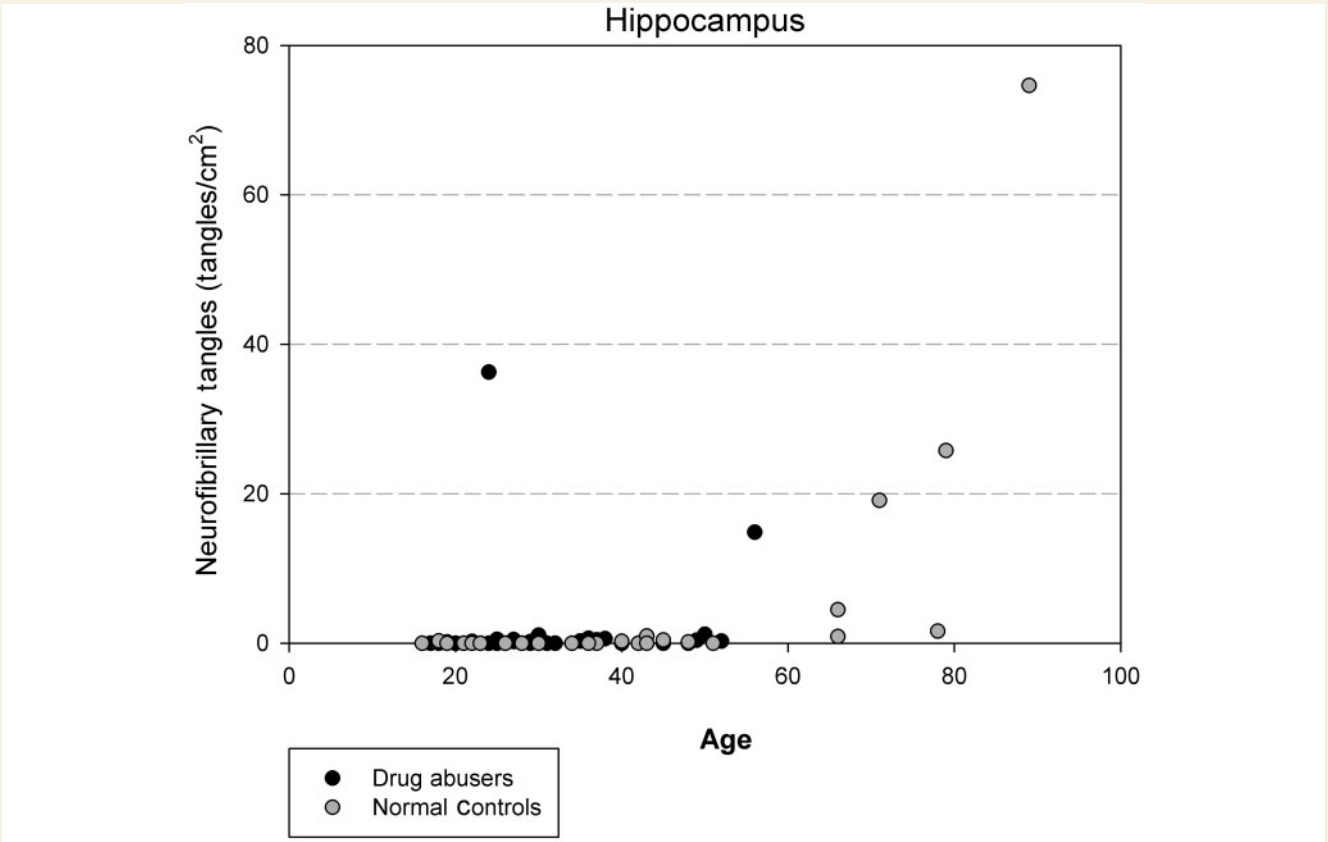

Figure 6 Correlation between number of neurofibrillary tangles and age in both drug abusers and control subjects. No direct correlation was observed in either group. However control subjects below the age of 60 years all had <1 neurofibrillary tangle per cm^2 while all control subjects over the age of 60 years had >1 neurofibrillary tangle per cm^2.

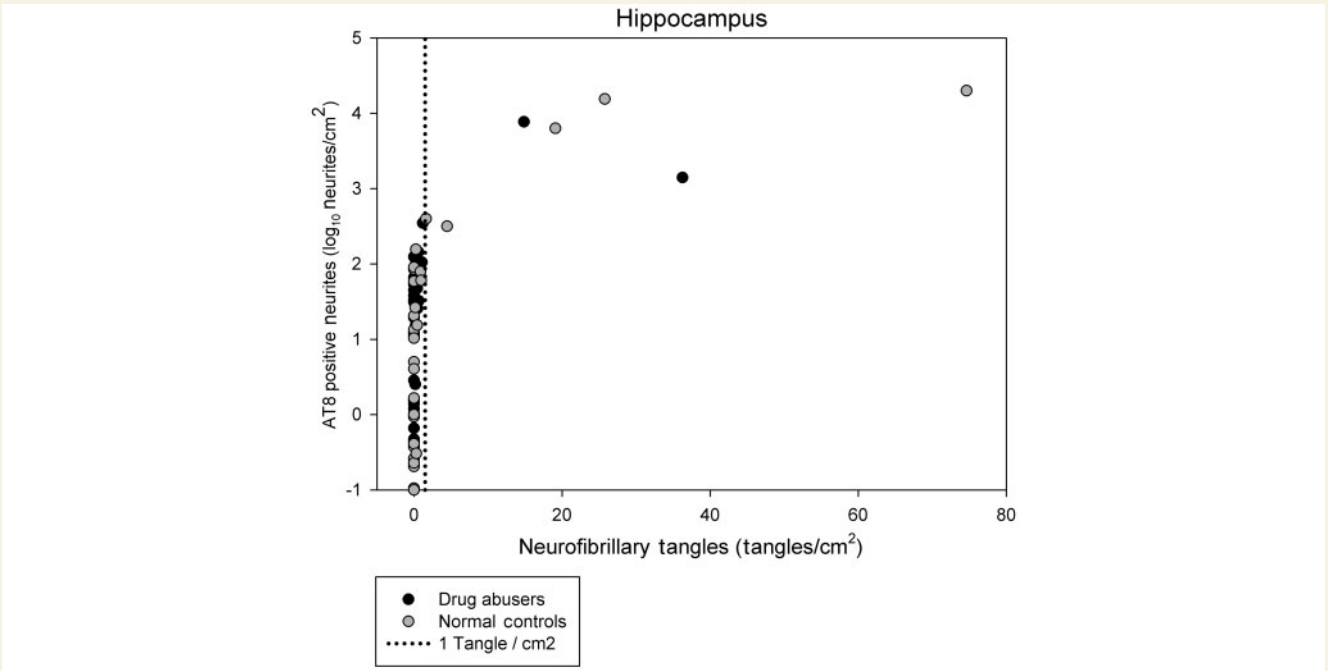

Figure 7 Correlation between quantitation of neurofibrillary tangles and neuropil threads in drug abusers and controls subjects in the hippocampus. The number of neurofibrillary tangles exceeded 1 per cm^2 only in brains that displayed more than 200 (or $10^{2.3}$) neuropil threads per cm^2 in both drug abusers and controls. (Note: logarithmic scale used.).

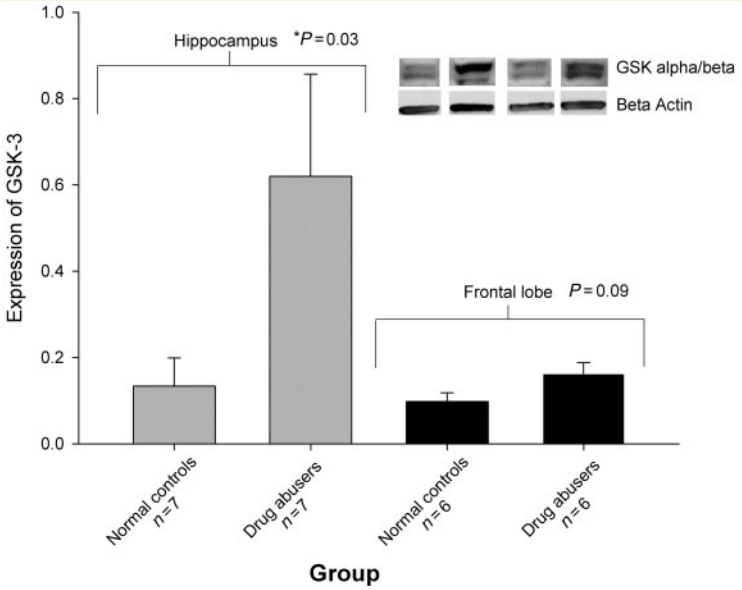

Figure 8 Western blot for GSK-3 expression in the hippocampus and frontal lobe of controls and drug abusers. A significant increase in the presence of GSK-3 protein was detected in the hippocampus of drug abusers ($P = 0.03$).

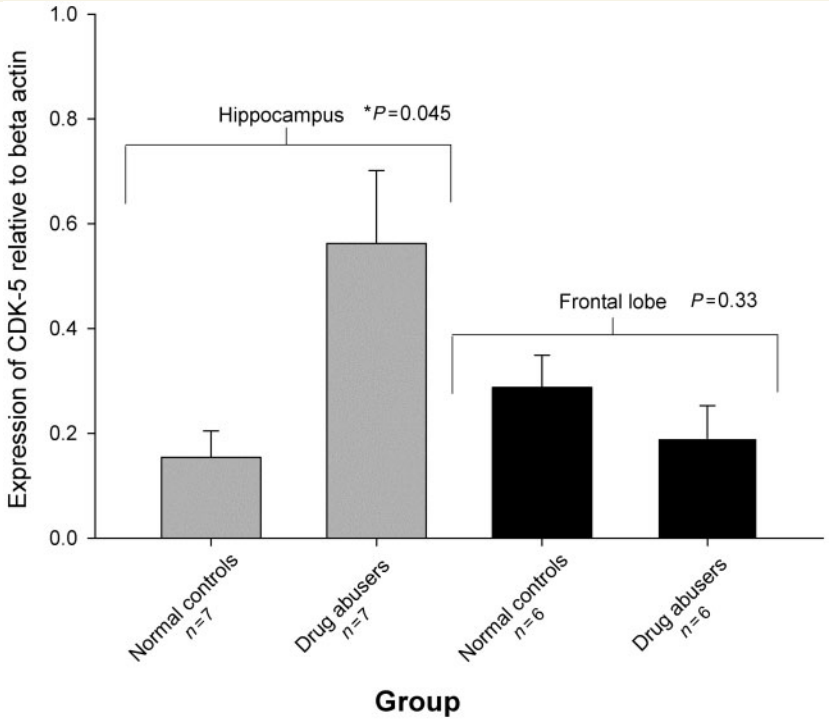

Figure 9 Western blot for CDK-5 expression in the hippocampus and frontal lobe of controls and drug abusers. A significant increase in the presence of CDK-5 protein was detected in the hippocampus of drug abusers ($P = 0.045$).

counts below which few neurofibrillary tangles are formed. When data points for neuropil thread quantitation in elderly control (non-demented) brains and for Alzheimer's disease brains are added to the comparisons (Fig. 5) it is possible to suggest a threshold level for neuropil thread load that is necessary for the clinical onset of dementia. This threshold lies between 2×10^4 neurites per cm^2, the highest level recorded in a non-demented case, and 1.9×10^6 neurites per cm^2, the lowest level observed in a case of Alzheimer's

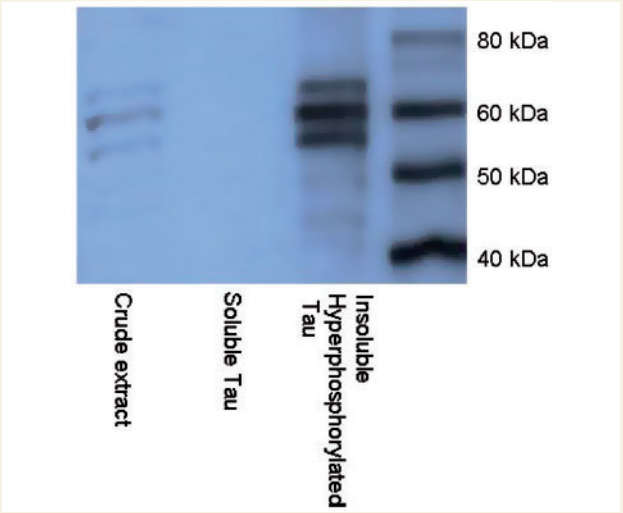

Figure 10 Soluble tau and hyperphosphorylated tau in Alzheimer's disease. Western blot for tau AT8 on unfractionated protein extract (crude), and fractionated soluble and un-soluble protein extract, demonstrating that AT8 positive proteins are located in the insoluble fraction.

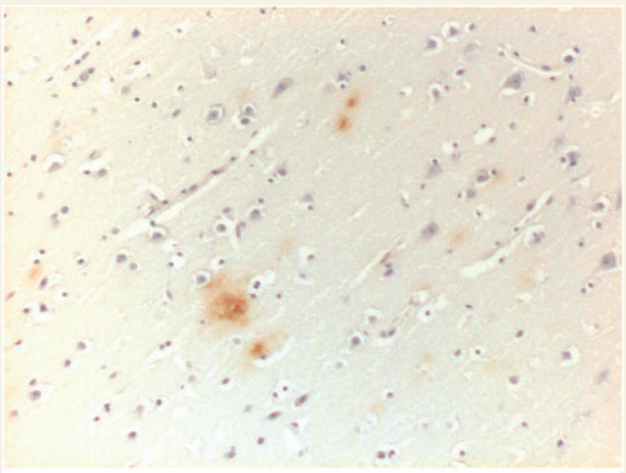

Figure 11 Staining for AB4 (beta amyloid) shows shadow plaques in the temporal cortex of a 30-year-old drug abuser.

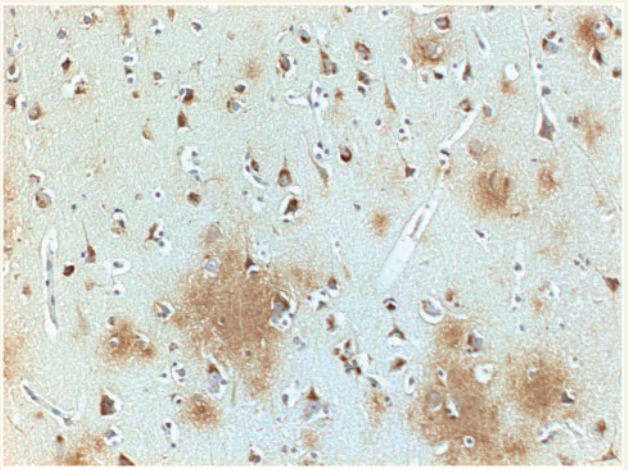

Figure 12 Staining of plaques and neurons in a drug abuser with 4G8 (beta amyloid).

disease. In all likelihood the clinical threshold will not be at the top end of this range as most subjects diagnosed with Alzheimer's disease live for several years with clinical symptoms, and assuming, therefore, that the pathology is progressive during the clinical phase of the disease, the threshold may be well below 1.9×10^6 neurites per cm^2. We recognize that this calculation ignores the role of neurofibrillary tangles and beta amyloid, but given our finding of a link between neuropil threads and neurofibrillary tangles (neuropil thread threshold at $\sim$200 per cm^2), and the recognized contribution of neuropil threads to dementia, we suggest that this quantitative assessment may be useful in understanding the early pathogenesis of dementias. We recognize a limitation in the study, in that we are unable to determine if tau degenerative changes are present in both the neurites and soma of individual cells and therefore the accumulation of both is truly linked. Nevertheless our data sheds new light on the process of neurodegeneration that will inform the direction of future studies.

To our surprise, we found no apparent link between hyperphosphorylated tau deposition and evidence of chronic hypoxic injury, although there was a significant association with breakdown of the blood–brain barrier. The association between cerebrovascular disease and Alzheimer's disease is now well-established (Esiri *et al.*, 1999; de la Torre, 2002).

The accumulation of insoluble tau protein disrupts normal neuronal function and eventually leads to neuronal cell death. The normal function of tau involves binding to, and stabilizing, microtubules. Expression of normal tau messenger RNA has been reported to be decreased in rats treated chronically with morphine, suggesting that a decreased turnover tau protein may afford greater opportunities for hyperphosphorylation to occur (Marie-Claire *et al.*, 2004). Tau phosphorylation and dephosphorylation is an important part of the normal biology of the neuron. The phosphorylation of tau is controlled by several kinases and phosphatases including GSK-3 and CDK5. We have demonstrated an elevation in the expression of GSK-3 and CDK-5 in the hippocampus of drug users. We recognize that measuring the presence of the enzyme does not necessarily reflect enzyme activity, but we observed a trend for high tau burden to be associated with higher expression of GSK-3. These data fit with reports from Liu *et al.* (2003) who demonstrated that overexpression of GSK-3 in the rat induced hyperphosphorylation of tau at multiple sites and was associated with impaired spatial memory. Activation of GSK-3 is reported to be an early event in Alzheimer's disease lesions, accompanying the formation of neurofibrillary tangles and other hyperphosphorylated tau inclusions (Leroy *et al.*, 2007).

The *ApoE* allele E4 has been linked in a number of studies to Alzheimer's disease and therefore the accumulation of both hyperphosphorylated tau and beta amyloid in the brain (Corder *et al.*, 1993). In this study no direct association between E4 and elevated

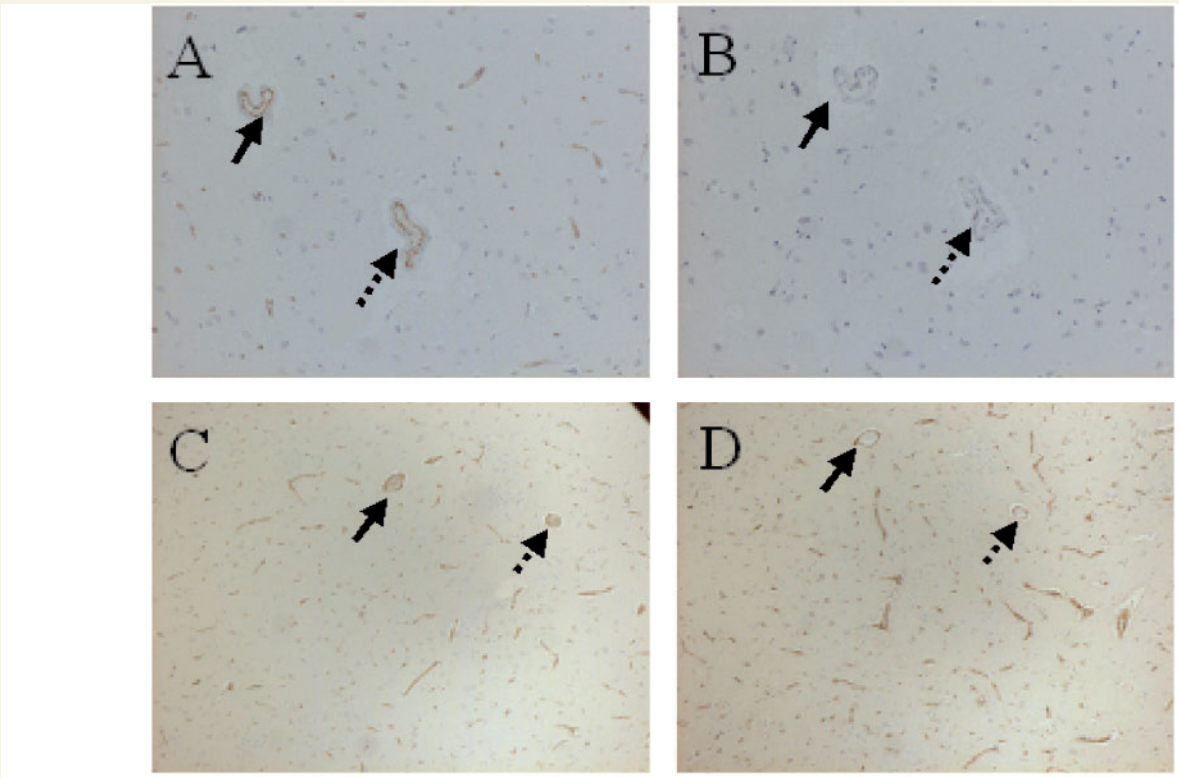

Figure 13 Von Willebrand endothelial cell (**A** and **C**) and ZO-1 tight junction protein (**B** and **D**) immunostaining. **A** and **B** demonstrate tissue sections with high levels of ZO-1 tight junction staining, in comparison **C** and **D** show tissue with virtually no ZO-1 immunoreactivity.

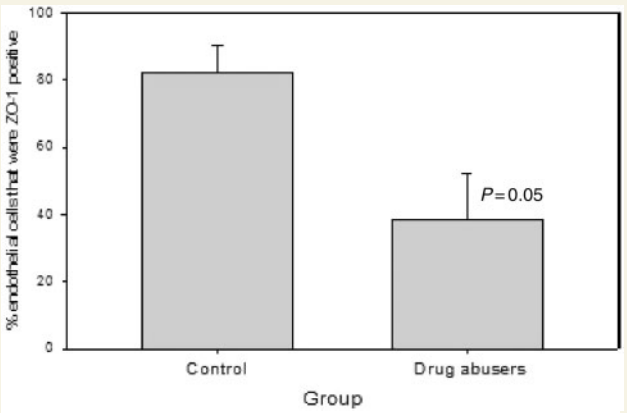

Figure 14 Comparison of blood–brain barrier tight junction integrity in drug users and age-matched control subjects. Structural integrity was determined by staining sections with an endothelial cell marker and then quantifying the percentage of blood vessels that also expressed detectable levels of the tight junction protein ZO-1. Blood–brain barrier tight junction disruption was significantly increased in drug abusers ($P = 0.05$).

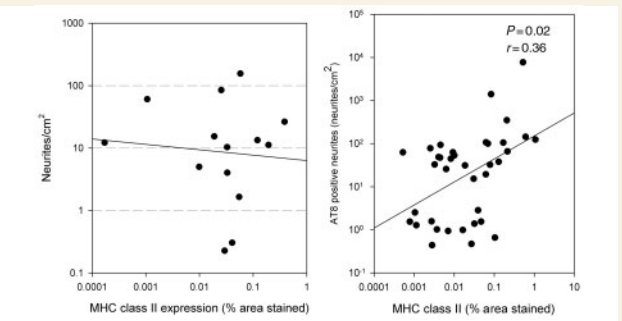

Figure 15 Correlation between hyperphosphorylated tau and major histocompatibility complex class II expression in the hippocampus of drug abusers and control subjects. No correlation exists in non-drug abusing age-matched control subjects, however major histocompatibility complex class II expression did correlate with the presence of hyperphosphorylated tau in drug abusers ($P = 0.2$).

hyperphosphorylated tau levels was detected, although the number of cases studied and the relative frequency of E2 and E4 give this study low power in respect of discerning a direct role for any *ApoE* allele.

This study shows that drug use accelerates the natural ageing process of the brain, possibly driving the brain towards an Alzheimer's disease-like pathology and reducing an already challenged brain reserve capacity (Fein and Di Sclafani, 2004). Whether the changes represent early Alzheimer's disease or another form of tauopathy is unclear, since the deposition of

hyperphosphorylated tau in the entorhinal and frontal cortices in these drug users does not fit with early pathology in the Braak staging system (Braak and Braak, 1991). It is also possible that these changes are potentially reversible in young drug users. Studies in a mouse tauopathy model showed that young mice were able to clear hyperphosphorylated tau deposits unlike older animals (Dickey *et al.*, 2009). However, our findings of increased hyperphosphorylated tau with age suggest that drug users are not clearing hyperphosphorylated tau effectively.

We are unable to determine whether or not the level of drug intake influences the deposition of hyperphosphorylated tau. While it is a tempting assumption, obtaining accurate data from subjects during life is notoriously difficult, they often have chaotic lives, unreliable memories and inaccurate reporting of illegal activities.

The most significant correlate for hyperphosphorylated tau deposition proved to be the level of microglial activation. Whether changes in microglia are merely reactive to the rise in hyperphosphorylated tau, or to some other subtle pathology in drug-user brains, is unclear, but the parallel with Alzheimer's disease pathology is noted (McGeer and McGeer, 1999; Cagnin *et al.*, 2006). These data complement that of other investigators who have demonstrated cognitive decline in opiate abusers and who predicted that pathology in drug users is centred in the frontal and temporal cortices (Ersche *et al.*, 2006), and provide a pathological basis for these observations.

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