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Short communication

Assessment of neuronal density in the putamen in human immunodeficiency virus (HIV) infection. Application of stereology and spatial analysis of quadrats

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Human immunodeficiency virus causes neuronal loss in various brain regions, but it has not been reported in the putamen. However, decrease in the volume of the putamen has been observed by magnetic resonance imaging. In order to clarify this issue two complementary methods: the stereological probe, the disector, and spatial analysis of quadrats, were applied in non-demented individuals who had died of acquired immune deficiency syndrome. A 21% decrease in neuronal density was observed in the human immunodeficiency virus group, especially those cases with human immunodeficiency virus encephalitis; however the statistical significance of this finding was borderline.

Keywords: HIV; putamen; spatial analysis; analysis of quadrats; neuronal clustering; neuronal loss

Acquired immune deficiency syndrome (AIDS) is often associated with cerebral disease. Neuropathologically, apart from opportunistic infections and neoplasms, human immunodeficiency virus (HIV) causes encephalitis and leukoencephalopathy (Budka *et al*, 1991). The encephalitic lesions are prominent in the deep grey and white matter, whilst rare in the cortex. Recently, quantitative studies have substantiated neuronal loss in AIDS, occurring independently of encephalitis in the neocortex, hippocampus, substantia nigra, and the cerebellum (Everall *et al*, 1993). This loss may be a component of the pathological substrate of the clinically observed cognitive, behavioural and motor abnormalities of the HIV-associated dementia complex (HAD). However, basal ganglia functions, which include sensorimotor aspects of movement, movement planning, motor memory storage and retrieval (Graybiel, 1990), may also be disrupted in HAD. The absence of aphasia, apraxia and agnosia in HAD are thought to indicate a subcortical process, and quantitative magnetic resonance imaging (MRI) has, using the Cavalieri theorem, reported reduced putamen volume in patients with HIV associated dementia. This was not reported in non-demented AIDS or symptom-free individuals (Aylward *et al*, 1993). It was proposed that this diminution was due to neuronal loss. In order to assess whether there is such loss in the putamen, we applied the disector probe and analysis of quadrats (Zahl, 1974). This facilitated both an accurate three-dimensional examination of neuronal density, and information regarding its pattern of arrangement.

The numerical density for both neurons and non-neuronal cells in the HIV and control brains are shown in Table 1. There was a decrease of 21% in the neuronal number, from 396 ± 133 ($\times 10^2$ mm^{-3}) in the control group, to 316 ± 87 ($\times 10^2$ mm^{-3}) in the HIV group. This was significant only with a one-tailed Student's *t* test ($P < 0.05$), which is appropriate when the change can only be in one direction, that is neuronal loss. On subdividing the HIV cases into those with HIV encephalitis and those with non-specific changes, the majority of the decrease, 27%, occurred in the encephalitis group, 288 ± 87 ($\times 10^2$ mm^{-3}), compared to 348 ± 84 ($\times 10^2$ mm^{-3}) in the non-specific group. Again the differences between cases with HIV encephalitis and controls was only significant with a one-tailed Student's *t* test ($P < 0.04$). Regression analysis failed to show correlation between the neuronal and non-neuronal

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Table 1 The numerical density (X10^2 per mm^3) of neurons, and other non-neuronal cells excluding endothelial cells, in the HIV and control groups. Cases H1–H6 and H13 are HIVE, H7-H12 are HIVM. In addition, the number of occurences of GFAP-positive cells for each case are listed

HIV Case	*Neurons*	*Non-neuronal cells*	*GFAP-positive cells*	*Controls Case*	*Neurons*	*Non-neuronal cells*	*GFAP-positive cells*
H1	296	1536	a	C1	664	1400	b
H2	296	1464	a	C2	244	1308	c
H3	264	1344	b	C3	260	1316	d
H4	288	1416	a	C4	368	1024	d
H5	392	1144	b	C5	416	1432	d
H6	120	1864	b	C6	296	1184	c
H7	408	1248	d	C7	448	1048	d
H8	368	1456	d	C8	360	456	c
H9	272	1320	d	C9	512	1672	c
H10	232	896	c				
H11	456	1424	c				
H12	352	1112	d				
H13	360	1296	a				
Mean	316	1348			396	1204	
s.d.	87	233			133	345	

s.d. = standard deviation; [a] = numerous occurences of GFAP-positive cells; [b] = moderate occurences of GFAP-positive cells; [c] = few occurences of GFAP-positive cells; [d] = no GFAP-positive cells

cell densities for either the HIV group (r^2 = 0.13) or the control group (r^2 = 0.16), indicating that the neuronal density was not related to sampling occurring mainly in sites with white matter tracts. As shrinkage was equal between the two groups this also could not explain differences in neuronal density for the two groups. In addition, all cases with numerous occurrences of GFAP-positive cells were those with HIV encephalitis, and the HIV group had more GFAP-positive cells compared to controls ($P <$ 0.005, Wilcoxon Rank-Sum two-tailed test).

Table 2 shows the number of cases which display statistically significant (F ratio $P < 0.01$) neuronal clustering at various block sizes in the two groups. However, multiple analysis of variance taking into account various block sizes, individual cases, and segregation into different groups, did not reveal any significant difference either in the number of clusters or the cluster frequency at a particular block size between the HIV and control groups, or between three groups: HIV encephalitis, HIV non-specific, and controls.

This study has demonstrated that statistically there is equivocal evidence that neuronal loss may occur in the putamen in non-demented individuals with AIDS. The neuronal decrease in the HIV group, which was larger in those with encephalitis, was significant only with a one-tailed t test. The use of such a test is valid in circumstances where a change can only occur in one direction, as in neuronal loss. Two potentially confounding variables, tissue shrinkage and inadvertent sampling of white matter tracts in the putamen were accounted for. Tissue shrinkage, due to disease or processing was assessed and found to be the same across the groups, while the lack of correlation between neuronal and non-neuronal cell densities indicated that white matter tract sampling did not account for differing neuronal densities. Furthermore, even though the mean age of the control group appeared to be older it did not differ from the HIV group in either brain weight or tissue shrinkage.

Importantly, the quadrat analysis did not reveal any clustering differences between the two groups, implying that if loss had occurred it was either random or not of sufficient degree to alter substantially the neuronal pattern. The equivocal evidence for decreased neuronal density is consistent with the neuroimaging study by Aylward *et al* (1993) who observed that the volume of the putamen was unaffected by HIV infection but was reduced in individuals with HAD. However, clinicopathological correlations regarding the relationship between neuronal

Table 2 The number of cases displaying statistically significant ($P < 0.01$) neuronal clusters at different block sizes in each group

Block size (mm^2)	*Number of clusters NIV*	*Controls*
0.005	0	0
0.01	1	1
0.025	9	7
0.05	5	1
0.0625	0	0
0.125	11	7

Two fields have an area of 0.005mm^2. The mean cell count for each block size is determined, and its variance compared to the variance of the smallest block size, which is equal to one field. With a random arrangement the variance is unaffected by block size change. A significant variance alteration, from the F-ratio ($P < 0.01$) of the variance, indicates neuronal clustering at that block size.

loss or pattern cannot be established from this present pathological study, as none of the individuals had been prospectively assessed for neuropsychological impairments. Such correlations require appropriate quantiative methodology as well as accurate clinical information and the utilisation of retrospective case note data can be unreliable.

Neuronal loss within the putamen would be expected to affect one or several neurochemically distinct populations. The predominant endogenous neurotransmitter in the putamen is gamma-aminobutyric acid (GABA), and the main afferent neurotransmitter, from the cortex, is glutamate. The mechanism by which HIV affects neuronal damage is not yet clarified. Indirect mechanisms of neurotoxicity are postulated to involve macrophages, microglia and astrocytes, due to cytokine production, resulting in neurotoxicity (Everall *et al*, 1993). Nonetheless, the involvement of cytokines in producing neuronal damage and therefore cognitive deficits has yet to be substantiated. They have been found to be increased in patients who died of AIDS, but there was no correlation with dementing features (Taylor *et al*, 1992).

In vitro the HIV envelope glycoprotein gp120 is neurotoxic (Brenneman *et al*, 1988); it activates calcium channels including those linked to glutamate (Lipton, 1991), resulting in a rise in intracellular calcium followed by neuronal death. This can be blocked by calcium channel antagonists. *In vivo* quinolinic acid, a glutamate agonist, is elevated in the cerebrospinal fluid of patients with AIDS with cognitive and motor abnormalities (Heyes *et al*, 1991), implying that an endogenous excitotoxin is involved in neuronal damage and death. Moreover, gp120 causes cognitive and motor impairments in neonatal rats (Hill *et al*, 1993).

In conclusion, this investigation in applying two independent techniques, has failed to substantially demonstrate neuronal loss in the putamen in non-demented individuals. Assessment of neuronal density in clinically prospectively studied individuals with dementia would clarify whether there is an associated significant reduction in the putamen. Furthermore, indices of synaptic and dendritic damage, such as staining with synaptophysin and MAP-1, could be assessed to clarify whether this predates neuronal loss. The importance of such an investigation would be twofold. Firstly, it would confirm whether neuronal loss underlies the reduced putamen volume observed in demented patients (Aylward *et al*, 1993), and secondly, whether this neuronal loss has a direct correlation with clinical features. Currently, as cortical neuronal loss does not correlate with the severity of dementia (Everall *et al*, 1994) the pathological substrate of cognitive dysfunction remains obscure.

Procedures

Thirteen AIDS cases (H1–H13) were examined, with no evidence of either opportunistic infections or neoplasms, who had either HIV encephalitis (cases H1–H6 and H13), characterised by the presence of multinucleated giant cells (Budka *et al*, 1991), or non-specific pathological findings including mild perivascular inflammatory cell cuffing, or astrocytosis. The cases were obtained from the MRC National AIDS Neuropathology Database and Central Brain Tissue Bank, Department of Neuropathology, Institute of Psychiatry. The mean age of the group was 36.2 ± 8.2 years, with an age range of 22–48 years. Clinical data, acquired retrospectively from case note analysis, revealed that 12 of the individuals died with advanced HIV disease, while case H12, who was previously well, died suddenly of septicaemia, secondary to streptococcal pneumonia. None of the thirteen cases had documented clinical evidence of HAD.

Nine control cases, mean age 45.3 ± 12.8 years and age range 20–60 years, were examined for comparison (cases C1–C9). All the control cases were male and they died of a variety of systemic illnesses including myocardial infarction, aortic aneurysm, gastrointestinal haemorrhage, bronchopneumonia, and suicide. Case C2 died of a pontine haemorrhage; however, this was limited to the cerebellum and pons, while the cerebrum was unaffected. Apart from this case, none of the control cases had evidence of any gross neuropathology on examination of the brain.

Tissue blocks from the putamen, at the level of the anterior commissure, were embedded in paraffin-wax, sectioned at 20μm, and stained with cresyl violet as previously described (Everall *et al*, 1991). Prior to processing the tissue blocks were photographed with a rule. The surface area of this block was compared with the surface area of the mounted section to estimate the degree of shrinkage during processing. Tissue shrinkage did not differ between the two groups. Furthermore, there was no difference in the mean fixed brain weights between the two groups.

Estimation of the neuronal numerical density was performed using the optical disector (Everall *et al*, 1991), which is a three-dimensional unbiased stereological probe specifically sensitive to estimating cell number. Neurons were identified by a clear nuclear profile containing a nucleolus and Nissl substance in the cell body. All other cells, excluding endothelial cells, were recorded to ensure that neuronal density estimation was not affected by sampling an area that was mainly composed of a white matter tract. The starting point for the estimation was random in each case. The measuring grid thereafter was applied contiguously with fields of quadrats examined as a 10 × 10 grid. This grid arrangement allowed analysis, by the Greig-Smith procedure, of whether neurons were random or clustered (Zahl, 1974). The technique repeatedly calculates the variance of the neuronal counts

throughout the quadrats as these quadrats are successively merged to form larger blocks. The variance does not change if the neurons are randomly arranged but a significant alteration in the variance occurs with clustering, as identified by the F-ratio (Zahl, 1974), at a block size similar to a cluster size.

Consecutive sections were stained immunocytochemically for glial fibrillary acidic protein (GFAP) for astrocytes using the avidin-biotin complex reaction. These were rated semiquantitatively, blind to the diagnosis, as having either numerous, moderate, few, or no occurances of GFAP positive cells.

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