

Stereological approaches to dementia research using human brain tissue

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## 1. Abstract

The relationship between the clinical features of dementia disorders and the resultant changes in underlying neuropathological mechanisms has long been of interest to researchers working in the field of neurodegenerative disorders. The majority of neuropathological research in dementia has utilized semi-quantitative analysis of protein inclusions, which have defined the hallmark histological features of the conditions. However, the advent of three-dimensional stereological techniques has enabled unbiased and fully quantitative assessment of brain tissue. The present review focuses on studies that have used these techniques to elucidate important relationships between neuropathological changes and clinical features and, in doing so, revealed important mechanistic insights into the pathophysiology of dementia disorders.

## 2. Introduction

Understanding the neuropathological correlates of the neurodegenerative dementias has remained a subject of intense interest and scrutiny over many decades. Neurodegenerative processes involve cascades of pathological events that culminate in the progressive and irreversible dysfunction and destruction of neurons and synapses, spreading in a stereotypical fashion through regions of the brain, defining its clinical presentation (Jellinger, 2001, Jellinger, 2012). The spectrum of dementing neurodegenerative disorders are usually bound by a common pathological factor, namely the presence of aberrant misfolded intra- and extra-cellular deposits of native proteins (Hardy and Gwinn-Hardy, 1998, Hyttinen et al., 2014). The neuron populations and brain areas affected are generally defined by the protagonist protein, thus characterizing clinical symptomatology (Taipa et al., 2012).

In large, the findings that have defined the major neuropathological markers associated with neurodegenerative dementias have stemmed from semi-quantitative 'two-dimensional' methods. Indeed, all major staging criteria in neurodegenerative dementias recommend single section sampling of markers in specific anatomical regions deemed vulnerable to pathological changes in the brain and brainstem (Braak and Braak, 1995, Montine et al., 2012, Thal et al., 2002, Braak et al., 2004, McKeith et al., 2005). Whilst this method is widely used the classification of diseases in post-mortem tissue, this approach is undermined by factors arising from bias in the interpretation of three-dimensional structures and markers on two-dimensional sections taken from one part of a particular reference area. Stereological analysis, on the other hand, describes a methodological approach that provides a three-dimensional interpretation of structures based on observations made on two-dimensional sections, representing the 'gold standard' for the unbiased assessment of the structural components of the brain. This means that the strict sampling protocol that stereology requires is often at odds with the need for the rapid and efficient

classification of cases, where a balance must be met between diagnostic and research requirements.

Despite the difficulties in incorporating stereological protocol into analysis of pathological changes, the inherent benefits of the approach have encouraged researchers to seek out the often subtle changes to the human brain across the various dementing disorders. This review draws together a plethora of diverse stereological studies that have mapped morphological changes in the brain, such as in neuronal populations or vascular integrity, with ante-mortem clinical and/or post-mortem pathological correlates.

### 3. The benefits of stereological analysis in post-mortem human brain tissue analysis

The problems arising from two-dimensional analysis, including issues surrounding the 'reducing fraction', widely used 'correction' formulae, such as that devised by Abercrombie, and the 'reference trap', arising from the use of density as a proxy measure of number, have been well documented elsewhere (Clarke, 1992, Hedreen, 1998) and beyond the scope of this review. However, despite the inherent difficulties in conducting three-dimensional analysis in post-mortem human brain tissue, well-designed and executed stereological studies have been performed. The findings emanating from such studies have yielded novel, interesting and, importantly, accurate, findings in field of dementia research.

A properly conducted design-based stereological study should be free of methodological ambiguity, requiring meticulous planning and preparation prior to commencing any experiment. Firstly, a clearly defined reference volume ( $V_{ref}$ ) should be defined for sampling in its entirety. The entire reference space can be systematically uniformly randomly sampled in the z-axis within serially sectioned structures or from blocks of tissue of a defined thickness. In the case of larger structures, such as neocortical regions, blocks of tissue may be acquired and sections taken at precisely defined intervals (e.g. 5mm to 1cm) (Fabricius et al., 2013, Pelvig et al., 2008). For smaller structures, such as brain nuclei or glands, serial sectioning may be more appropriate. In both cases, a uniform, random starting point should be selected within the first interval, followed by a complete series of sections at equally spaced intervals through the entire  $V_{ref}$ . Uniform, systematic sampling should also be applied to the  $V_{ref}$  on sections in the x and y axes, through the use of a probe relevant to the parameter measured of a specific size and depth, placed randomly and uniformly through the entire reference space at intervals, reflecting the desired level of accuracy of the population estimate from taking coefficient of error (CE) levels into account. The  $V_{ref}$  may be calculated using Cavalieri's formula:

$$V = T \cdot a(p) \cdot \sum P,$$

where  $T$  is the slab thickness or intersectional distance,  $a(p)$  is the area per point, and  $\sum P$  is the sum of points hitting the reference area.

Using the  $V_{ref}$ , calculated from Cavalieri's formula, one may then estimate the total number, length or surface area of objects per  $V_{ref}$  using a suitable probe. Most of the studies included in this review have used the optical disector approach (Gundersen et al., 1988) for the calculation of total neuron number, which uses the following formula:

$$Nv = \frac{\sum p^- Q^-}{P \cdot V}$$

where  $Nv$  is the numerical density,  $p^-$  is the disector samples,  $Q^-$  is the  $Q^-$ -weighted number of objects counted,  $P$  is the total number of disectors, and  $V$  is the disector volume.

A number of studies reviewed have also employed the physical disector (Sterio, 1984) approach to calculate synapse (Scheff et al., 2013, Scheff et al., 2011) and neuron (Pakkenberg et al., 1991) number. Here, pairs of thin sections are taken at intervals through the  $V_{ref}$  and the objects observable in the first, but not second, section of the pair are counted. This approach is useful when objects are large relative to section thickness, lending itself particularly well to images acquired from electron microscopy (Scheff et al., 2013, Scheff et al., 2011).

Alternatively, some studies reviewed (Joelving et al., 2006, Piguet et al., 2011) have employed the optical fractionator (Gundersen et al., 1988). Here, the total number of objects can be calculated by combining the optical disector with a systematic uniform sampling scheme, the fractionator. The optical fractionator estimates the total number of particles ( $N$ ) of the  $V_{ref}$  obtained by multiplying the reciprocals of the fractions with the total particle count ( $\sum Q^-$ ) per brain structure obtained within the optical disectors:

$$N = ssf^{-1} \cdot asf^{-1} \cdot tsf^{-1} \cdot \sum Q^{-1},$$

where  $ssf$  is the section sampling fraction,  $asf$  is the sampling fraction,  $tsf$  is the thickness sampling fraction and  $\sum Q^-$  is the total number of objects counted within the disector.

As most post-mortem tissue is cut in a non-random fashion in a vertical, uniform random manner, length and surface area measures could potentially be biased as a result of their orientation in the brain. For example, in the measurement of neuronal white matter tracts, the relationship between probe and the structure may be highly anisotropic, leading to inaccurate estimates of length. As such, reviewed studies examining the length of structures, e.g. neuropil threads (Giannakopoulos et al., 2007) have employed an intrinsically isotropic probes such as the cycloid or spherical 'space ball' (Mouton et al., 2002) probes.

#### 4. Alzheimer's disease

Alzheimer's disease (AD) is the most prevalent form of age-related dementia, accounting for approximately 70% of cases (Reitz and Mayeux, 2014). Clinically, AD is marked by memory loss and impaired reasoning with a gradual onset and a progressive course (McKhann et al., 2011). Neurofibrillary tangles of phosphorylated tau protein within neurons and extracellular plaques consisting of amyloid- $\beta$  protein are considered to be the characteristic neuropathological findings in AD upon post-mortem histological examination (Montine et al., 2012). Neurofibrillary tangles and amyloid- $\beta$  plaques are encountered at pathological predilection sites at early and pre-symptomatic stages, prior to stereotypical sequences of accumulation and deposition in vulnerable brain regions (Stratmann et al., 2015)

The hallmark pathological lesions in AD are also typically found in the elderly non-demented (Tomlinson et al., 1968, Price et al., 2009, von Gunten et al., 2010, Schneider et al., 2009), which has prompted speculation that AD is linked to senescent mechanisms in the brain (Herrup, 2010, Yankner et al., 2008). To investigate the link between senescence and AD, stereological studies have evaluated patterns of neuronal loss with aging in the absence of dementing symptoms, and compared these findings to neuronal loss typically observed in AD. West et al (1994) used the point counting method and optical disector probe to estimate neuronal number in subregions of the hippocampal formation and found age-related reductions in neurons in the CA4 and subiculum in controls ranging from 13 to 101 years. However, CA1 neuronal loss was found to be distinct to AD cases and not found in normal aging cases, suggesting specific patterns of neuronal loss in AD and that progressive aging alone cannot fully explain the pathological changes of AD (West et al., 1994).

Several staging schemes have illustrated the pathological progression of the characteristic lesions of AD (Braak et al., 2006, Thal et al., 2002, Josephs et al., 2014). Stereological designs have been used to study sites that are affected at early stages of the pathological process, often by comparing cases that had different levels of cognitive ability prior to death. These studies have often benefitted from excellent clinical information that can be compared to any observed cytoarchitectonic changes that may occur in tandem with cognitive dysfunction. However, some degree of caution must be exercised when comparing clinical information obtained during life with post-mortem findings. AD is marked by progressive deterioration meaning that control of the interval between the last clinical assessment and death is of great importance.

Assessment of whole brain hemispheres at systematic intervals from slabs using region point counting with the Cavalieri principle to determine volume of the neocortex and central grey matter (basal ganglia, thalamus, hypothalamus and substantia innominata) has shown volumetric reductions in the neocortex in AD cases when analysed as a whole. Parcellation into individual lobes showed significant reductions

in the occipital lobe only (Regeur, 2000). However, when the cohort was stratified based on severity of cognitive dysfunction, neocortical and subcortical grey matter structures decreased in volume with progressive clinical severity. Shrinkage following fixation in formaldehyde has long been known exist in post-mortem brain tissue (Mouritzen Dam, 1979). This study did not note the exact fixation period, beyond being greater than five months, thus introducing the possibility of fixation-related bias

A more region-specific approach has been used to investigate pathological predilection sites, such as the CA1 sector of the hippocampus and layer 2 of the entorhinal cortex, which are both affected at early stages of the pathological progression of AD (Thal et al., 2002, Braak et al., 2006). Post-mortem assessment of these regions was compared with the clinical dementia rating scale (CDR) to assess structural changes related to clinical phenotype (von Gunten et al., 2006). The entire hippocampus was sampled coronally by cutting into slabs along the anterior-posterior extent of the hippocampus. Sections were acquired from each slab at intervals and volume estimated using Cavalieri's method. Using the optical disector, a loss of neurons was found in the CA1, even at the earliest evidence of cognitive decline on the CDR, and that this progresses linearly with increasing cognitive decline. The entorhinal cortex showed a similar pattern, though a more marked loss of neurons was found in CDR level 1 (the lowest level of impairment). Regression models for the quantitative measures of neuropathological lesions in these regions showed that neurofibrillary tangles, but not amyloid- $\beta$ , significantly predicted the loss in neuron numbers. However, neurofibrillary pathology only partially predicted the degree of neuron loss in these models, leading the authors to speculate that more downstream mechanisms are also responsible for the degeneration observed.

Another stereological study of the entorhinal cortex also aimed to investigate the early process of neurodegeneration in AD (Gomez-Isla et al., 1996a) by assessing differences in neuronal number between progressively aged control cases and cases at various stages of cognitive impairment, based on CDR scores. To sample the entire entorhinal cortex, the structure was serially sectioned before taking five sections from a random starting point, with a 3 mm interval between each. Volume was determined using Cavalieri's method and neuronal density was estimated used the optical disector. Increasing age was not found to be related to any changes in number or density of neurons or the total volume of the entorhinal cortex. However, neuronal loss was found in all layers in AD, when compared to controls, but was especially severe in layer 2, with cases at the mildest stages of cognitive impairment showing significant loss in layers 2 and 4. The authors suggest that the dramatic loss at the earliest stages of cognitive decline, with no losses as a result of age, provide evidence for AD being a disease entity that is separate from an acceleration of the process of aging. When assessing findings from the entorhinal cortex, it should be noted that the cellular populations of the entorhinal cortex are highly variable across cases both with and without neurological disease (Heinsen et al., 1996). This is especially important as the

number of cases used at certain levels was low, with only two CDR1 cases used in the former study.

As mentioned earlier, stereological methods have applications beyond ascertainment of the number of neurons per structure, and can be used to determine the number or size of neuropathological lesions within brain regions of interest. This approach has been used in the investigation of the hippocampal formation and the superior temporal gyrus, regions known to be vulnerable to AD pathology at early stages of disease (Braak et al., 2006). Studies in the superior temporal gyrus have conducted analyses of pathology burden (Gomez-Isla et al., 1996b) and neuronal number (Gomez-Isla et al., 1997). These studies have both obtained the volume of structures using Cavalieri's method and combined this with the density of either pathological lesions or neurons using a subsample of the cortex being assessed. These studies revealed correlations the now well established relationship between amyloid- $\beta$  burden and apolipoprotein  $\epsilon 4$  allele, as opposed to tau (Rigaud et al., 1999). Counts of neurofibrillary tangles have been compared with neuronal number estimates on adjacent sections of the superior temporal gyrus as well as the duration of illness (Gomez-Isla et al., 1997). However, the study also revealed a greater loss in neuron number than neurofibrillary tangles, suggesting clearance or only a small contribution of the protein to neuronal loss in AD (Gomez-Isla et al., 1997). Giannakopoulos et al (2007) employed a similar approach by uniformly, randomly sampling the entire hippocampal-entorhinal region and estimating the total number of neurons within the CA1 sector of the hippocampus and entorhinal cortex, and compared this to the number of neurons bearing neurofibrillary tangles, the number and volume of amyloid- $\beta$  plaques, and the length of neuropil threads using the cycloid probe (Giannakopoulos et al., 2007). This study found that amyloid- $\beta$  was not related to neuron number in either region but that neurofibrillary tangle number was negatively correlated with neuron number in the CA1 region. Neuronal losses in CA1 and the entorhinal cortex both correlated with CDR scores, as did neurofibrillary tangle number. Neuropil thread length was related to increasing CDR scores, but this effect was only mediated through their relationship to neurofibrillary tangles (Giannakopoulos et al., 2007).

Mild cognitive impairment (MCI) is considered to be an intermediate state between normal levels of cognition and AD (Morris and Cummings, 2005) and provides a useful group for inclusion in studies that wish to assess changes that occur at early stages of cognitive decline. The degeneration of the cholinergic system in AD, including the nucleus basalis of Meynert (NBM), has been postulated to be an important pathogenic event in the development of the intellectual decline that marks AD (Perry et al., 1977). Mufson et al, 2000 investigated whether a loss of neurons expressing trkA receptors may underlie NBM degeneration in AD, due to the role of trkA receptors in maintaining the viability of these neurons (Mufson et al., 2000). The NBM from one hemisphere from AD, MCI and control cases was serially sectioned into 18 series to determine the volume of the NBM using the Cavalieri method and total number of trkA immunoreactive neurons calculated by the optical disector. A significant decrease in

neurons expressing trkA receptors was found in AD and MCI cases, thus suggesting a reduction in trkA positive neurons in the NBM does not mark a transition from MCI to AD, as the MCI cases also showed reductions in neurons expressing trkA receptors (Mufson et al., 2000).

Studies examining sites affected early in the pathological process of AD have also used MCI cases to compare the clinical time-point at which such sites become affected by neuropathological change. Kordower et al (2001) estimated the neuron number in individual layers of the entorhinal cortex using the Cavalieri and optical disector approach. Neuronal loss and neuronal volumetric reduction was found in layer 2 of the entorhinal cortex in AD and MCI cases. No difference was found in neuron number between AD and MCI, suggesting that loss or reduction in the size of neurons in layer 2 of the entorhinal cortex does not underlie the transition from MCI to AD. However, loss of layer 2 volume was found between control and MCI, as well as MCI and AD, and such losses correlated with clinical measures of cognitive dysfunction obtained during life (Kordower et al., 2001).

The posterior cingulate gyrus is affected at an early stage in the progression of AD and its degeneration may predict cognitive decline on neuroimaging (Barnes et al., 2007, Johnson et al., 1998). Based on previous findings showing that synaptic pathology is an early event in AD, and correlates well the onset with cognitive decline (Scheff and Price, 2006), Scheff et al, 2015 investigated synapse number in the posterior cingulate gyrus in AD, MCI and controls. Electron microscopy was used to determine synapse number within the posterior cingulate gyrus using the physical disector on randomly determined sections containing a superimposed counting frame on the micrograph (Scheff et al., 2015). A reduction in total number of synapses was found in AD patients, compared to control cases, with MCI cases showing no significant differences from AD or control cases. Total synapse number also significantly correlated with MMSE score, suggesting that synaptic dysfunction is an early event in cognitive impairment in AD.

The precuneus is also affected by amyloid- $\beta$  pathology at early stages of AD (Rowe et al., 2007). Scheff et al (2013) thus employed the physical disector to examine the number of synapses in this region. Alternate slabs of the precuneus gyrus were serially sectioned and sections systematically randomly uniformly sampled from within slabs. The remaining slabs were then used for pathological analyses (Scheff et al., 2013). Synaptic density was determined by sampling random areas within layer 3 of the precuneus using electron microscopy. The total number of synapses in layer 3 were reduced in AD cases. However, these reductions did not relate to any change in cognitive function or to the degree of amyloid- $\beta$  burden in the precuneus. A related study (Scheff et al., 2011) using similar methodology but conducted in layer 3 of the inferior temporal gyrus showed significant reductions in synapse number in both AD and MCI when compared to controls, but no difference between AD and MCI. Volumetric measures of layer 3 of the inferior temporal gyrus suggested that AD and

MCI were significantly reduced when compared to controls but that AD cases were also significantly reduced compared to MCI.

An alternative approach in the elucidation of the temporal sequence of morphometric changes under neurodegenerative conditions is to use cases with varying degrees of neuropathological burden. Such an approach relies on the current staging schemes to accurately reflect the cognitive status of the individual and there is good evidence that they are good general indicators of cognition (Maderna et al., 2015, Price et al., 2009), with notable exceptions in the relationship between pathology and clinical phenotype (Gertz et al., 1998). Such studies, however, are particularly useful in identifying relationships between the spread of pathological lesions to different regions and the corresponding effects on cellular populations. One study employing this approach assessed the CA1 and subiculum in cases of varying degrees of global neuropathological burden, with the aim of identifying the stages at which cellular changes become prominent (Rossler et al., 2002). Region point counting using the Cavalieri method was used to estimate the volume and neuron number in subregions of the hippocampus. A loss of neurons in the CA1 region was found in Braak neurofibrillary stages 4 and 5, but not in Braak stages 1-3, or as a result of progressive age. There was a trend toward neuron loss in the subiculum in cases with Braak stage 5 pathology, when compared against cases at Braak stage 1. The authors suggested that this study provides evidence for neuron loss in a region- and AD pathology stage-specific manner.

A combination of pathological and clinical data has also been used to identify cases possessing significant pathological burden, but no clinical history of dementia. As mentioned earlier, the hippocampus shows a pattern of neuron loss that is specific to AD, specifically in the CA1 sector (West et al., 1994), occurring in higher levels of global neurofibrillary pathology burden (Rossler et al., 2002). An investigation comparing control and AD cases to 'preclinical AD' cases with high global pathologic burden, but no clinical history of dementia in subdissected and systematically sampled hippocampus reported no difference in neuron number in preclinical AD cases when compared against controls in any subregion of the hippocampus (West et al., 2004). This implies that neuronal loss in such regions may be related to the transition of the clinical threshold in AD. However, it should be noted that this study used 'preclinical AD' cases that were generally of intermediate AD pathologic change, ranging from Braak neurofibrillary pathology stages 2 to 4. As previously noted, changes have only previously been identified in Braak stages 4 and 5 in some of the regions measured (Rossler et al., 2002). Therefore, the inclusion of several cases with a Braak stage lower than 4 may have increased the variation in results and diminished the power of the study to identify a significant difference between 'preclinical AD' and AD.

Neuroinflammatory responses mediated through glia are thought to play a prominent role in the etiology of AD, meaning glia may represent a useful target in the investigation of changes resulting from AD-type pathology (Heneka et al., 2015). Pelvig et al (2003) conducted morphometric assessment of glia, as well as neurons,

from the entire cortical hemisphere, taking sections from the front of alternate slabs from a random starting point (Pelvig et al., 2003). Cavalieri's method was used to determine grey and white matter volumes, whilst the optical disector was employed to estimate neuronal and glial density. This study found that AD cases had no significant differences in terms of neuronal or glial number compared to controls. The total neocortical and temporal lobe volume was, however, significantly reduced in AD cases.

Cerebral white matter lesions are considered to be a risk factor for dementia. In the neocortex, stereological studies have shown a decrease in white matter volume by as much as 28% with progressive age (Pakkenberg and Gundersen, 1997). However, in AD, the volume of the white matter was found to be unchanged (Pelvig et al., 2003). Nevertheless, it has been suggested white matter changes may contribute to cognitive impairment in the elderly by slowing synaptic communication (Filley and Cullum, 1994). On this basis, one study assessed myelinated fiber length and the mean diameter and volume density of white matter fibers (Jorgensen et al., 2008) using needle biopsies taken from tissue after a point grid was placed on the tissue to estimate fiber length, volume and number. Biopsies were randomly orientated and sectioned and the number of profiles per unit area was determined using disector counting frames. AD cases did not differ from controls in any parameters tested (Jorgensen et al., 2008).

## 5. Parkinson's disease and dementia with Lewy bodies

Parkinson's disease (PD) is a common neurodegenerative disorder, occurring in approximately 1% of people over the age of 60, but increasing in prevalence with advancing age (de Lau and Breteler, 2006). PD is primarily a motor disorder that is characterized by rigidity, bradykinesia, unstable gait and resting tremor (Mutch et al., 1986). However, 24-31% of cases diagnosed with PD also have dementia (PDD) (Aarsland et al., 2005). PDD is marked by impairments to attention, memory, visuospatial function and executive faculties (Vasconcellos and Pereira, 2015). Dementia with Lewy bodies (DLB) is thought to be second most common form of degenerative dementia after AD, accounting for approximately 20% of dementia cases at autopsy (McKeith, 2000). Clinically, DLB manifests as a progressive decline in cognitive ability, with three core features of cognitive fluctuations, parkinsonism and visual hallucinations being considered characteristic features of the disorder (McKeith et al., 2005). PD/PDD and DLB are both pathologically characterized by the presence of ubiquitinated intracellular inclusions of aggregated  $\alpha$ -synuclein known as Lewy bodies (Spillantini et al., 1997). Like proteinaceous inclusions seen in disorders such as AD, the sequence of Lewy body pathologic spread has been characterized in these disorders to standardize neuropathological assessment (McKeith, 2006, Braak et al., 2004).

The clinical features of PD and DLB differ somewhat from those observed in AD. Whilst AD is typically marked by a progressive impairment in memory, this is not as clinically prominent in PD and DLB (McKeith et al., 1996). Therefore, one important avenue in PD/DLB research has been to investigate whether changes to the hippocampus, a structure involved in memory and consistently implicated in the pathophysiology of AD, are also found in PD and DLB. To investigate the hippocampus in DLB and PD compared to control cases, one study sampled the rostrocaudal extent of the hippocampus using coronal tissue slabs taken at uniform intervals (Harding et al., 2002). Volume and neuron measurements were conducted within the subdivisions of the hippocampus. A significant reduction in hippocampal volume was found in DLB cases, with specific atrophy present within the subiculum. Significant neuronal loss was only found in the subiculum of DLB cases, and PD cases showed no difference from controls in any parameter. Likewise, Joelsing et al (2006) used the optical fractionator to estimate neuronal number in the hippocampi of mostly cognitively impaired PD cases of during life against control cases. The hippocampus was subdivided from serial uniform coronal slices of the entire hemisphere and sections systematically, randomly sampled from the front of each block. No significant difference in neuronal or glial cell number, or grey matter volume, was found in any region analysed. The authors suggested the findings provided evidence of a degree of specificity regarding topography of sites affected by neurodegenerative changes in dementia disorders and that hippocampal degeneration is not invariant across the spectrum of neurodegenerative dementia disorders.

Neuroimaging studies of PD brains have previously shown cortical atrophy, suggesting cortical changes contribute to the manifestation of cognitive symptoms within this disorder (Becker et al., 1979). Additionally, PD brains typically show cortical involvement at the most severe stages of Lewy body pathology (Braak et al., 2004). Pedersen et al, 2005 used stereological methodology to investigate whether such changes to the cortex in PD were related to the number of neurons within the cortex (Pedersen et al., 2005). No change in total neuronal number or volume was found in PD cases in the neocortex. Furthermore an *increase* in neuron number was found in the temporal lobe of PD cases. This study thus implies that the volumetric reductions and Lewy body pathology previously reported in the cortex in imaging studies do not translate to changes to cortical neuron populations that PD cases.

The motor symptoms that characterize PD are thought to result from the degeneration of pigmented dopamine-producing neurons of the midbrain structure, the substantia nigra (Mettler, 1964). Stereological studies have thus examined absolute neuronal numbers in PD cases. To investigate the degree of nigral neuronal loss in PD, Pakkenberg et al (1991) serially sectioned the substantia nigra *pars compacta* and used the Cavalieri and physical disector methods to measure the volume of the structure, as well as the number of pigmented and non-pigmented neurons, respectively. PD cases had a substantial mean 66% loss of pigmented neurons

compared to controls and a mean 25% loss of non-pigmented neurons compared to control cases (Pakkenberg et al., 1991).

The thalamic nuclei are also affected by Lewy body pathology and have been implicated in impaired physiological functions in PDD and DLB such as arousal, vigilance and cognition. The intralaminar thalamic nuclei have been investigated by serial sectioning of the thalamus to the emergence of the pulvinar, and the sampling at uniform intervals in DLB, PD and PDD cases (Brooks and Halliday, 2009). A significant reduction in neurons in the centromedian/parafascicular, and volume in the paratenial, cucullar and central lateral nuclei, was found in DLB, PDD and PD cases, when compared to controls. A correlation between neuronal number and Lewy body burden was found in the centromedian/parafascicular nucleus. No difference was found in neuron number in demented cases compared to non-demented cases, though cucullar nucleus losses were greater in hallucinating cases when compared to non-hallucinating cases.

The phenomenon of complex visual hallucinations has previously been suggested to be related to changes to the visual system in DLB (Taylor et al., 2011). Accordingly the afferent visual system has been examined in DLB patients using stereological protocol. A major component of the afferent visual system, the lateral geniculate nucleus (LGN), was investigated by serially sampling specimens and determining area using the Cavalieri method and estimating total neuronal number using the optical disector (Erskine et al., 2015). No differences in neuron number were found in hallucinating DLB cases when compared to non-hallucinating AD cases. However, non-hallucinating AD cases were shown to have significant neuronal loss and gliosis (Erskine et al., 2015).

Respiratory disorders are often found in Lewy body disease and in other synucleinopathies such as multiple systems atrophy (Iranzo, 2007). In MSA, respiratory dysfunction is secondary to the autonomic dysfunction that marks the disorder, and typically subclinical in DLB (Iranzo, 2007). The pre-Bötzinger complex and the medullary raphe nuclei, which are thought to have a role in respiratory functioning respiration, have been investigated in MSA and Lewy body disease cases on the basis of this role in respiratory function (Presti et al., 2014). A significant reduction in neuron number was found in both regions in Lewy body disease and MSA cases, with a greater magnitude of reduction in MSA cases.

## 6. Huntington's disease

Huntington's disease (HD) is a motor disorder thought to result from a trinucleotide repeat expansion in the gene encoding huntingtin (Walker, 2007). Clinically, HD is marked by a variety of motor deficits including a distinct chorea, incoordination and slowed saccadic eye movements (Walker, 2007). In addition to motor symptoms, cognitive deficits are also frequently found, though they typically affect executive

function rather than memory (Brandt et al., 1988). Neuropsychiatric symptoms are often common and HD patients have high rates of depression and suicide ideation (Di Maio et al., 1993). Pathologically, the striatum is atrophic and accordingly the existing staging scheme for determining the degree of HD neuropathological change is based upon the degree of neuronal loss and atrophy in the striatum (Vonsattel et al., 1985). However, post-mortem histology for the protein huntingtin or ubiquitin also reveals intranuclear and cytoplasmic inclusions in striatal and cortical neurons (Sieradzan et al., 1999)

A focus of HD research has been the investigation of the striatum and basal ganglia, on the basis that deficits in these regions may underlie the well characterized motor deficits in this disorder (Vonsattel et al., 1985). Whilst three-dimensional stereological designs have not yet been used to study the striatum in HD, two-dimensional studies of neuronal density in single striatal sections have shown substantial reductions in HD when compared to control cases (Myers et al., 1991). Such approaches in other brain regions have also revealed cerebellar degeneration (Rub et al., 2013) and in the substantia nigra and pons (Rub et al., 2014). However, the cortex has also been implicated in HD, with two-dimensional densitometric studies showing reduced density of large neurons of layers three, five and six of the prefrontal cortex (Sotrel et al., 1991) and volumetric reductions in the neocortex (de la Monte et al., 1988). Considering the inherent problems of assuming that density acts as a proxy measure of total number, one study aimed to estimate neuronal number in the entire hemisphere of HD cases, using stereological methodology (Heinsen et al., 1994). The entire hemisphere was embedded and serially sectioned into 500 $\mu$ m-thick sections and stained with gallocyanin. Cortical and striatal total neuronal number were significantly reduced in HD compared to control cases.

Although motor abnormalities are well described in HD, other clinical phenotypes are frequently encountered. Stereological techniques have proved useful in obtaining quantitative data in the investigation of topographical changes underlying divergent clinical phenotypes. Mood predominant phenotypes of HD are also well described (Di Maio et al., 1993) and the heterogeneity of the clinical presentation between motor- and mood-predominant phenotypes may reflect heterogeneous patterns of neuropathological change. On this basis, two studies have assessed both the primary motor and the anterior cingulate cortices. The anterior cingulate is thought to be strongly implicated in mood disorders (Ebert and Ebmeier, 1996). These studies (Thu et al., 2010, Kim et al., 2014) used well defined subregions of the primary motor and anterior cingulate cortices, which were sampled into a systematically random series of sections. The total neuronal number could not be determined due to the tissue for the entirety of the structures being unavailable. Therefore, the volume of each subregion was determined using the Cavalieri method and neuronal density was estimated using the optical disector. Significant neuronal loss was found in both the primary motor and the anterior cingulate cortices in HD cases compared to controls. However, neuronal number varied considerably in HD cases, with those classified as 'motor-predominant'

showing more marked neuronal loss in the primary motor cortex and cases classified as 'mood predominant' showing greater neuronal loss in the anterior cingulate. Stereological analyses was also conducted on interneurons immunoreactive for the calcium-binding proteins parvalbumin, calretinin or calbindin. Selective significant reductions were found in calbindin immunoreactive neurons in motor-predominant HD cases, whereas significant reductions were found in parvalbumin, calretinin and calbindin immunoreactive neurons in mood-predominant HD cases. This study thus demonstrated the relationship between clinical features and neuropathological changes in the HD brain.

## 7. Frontotemporal dementia

Frontotemporal dementia (FTD) is clinically defined by behavioral changes, including changes in social conduct, disinhibition, loss of insight, apathy and emotional blunting (Faber, 1999). However, FTD is not heterogenous in terms of clinical features, with at least three subtypes (behavioral variant, semantic dementia and progressive nonfluent aphasia) recognized (Ghosh and Lippa, 2015). Overall, FTD is thought to be the third most common form of cortical dementia, accounting for approximately 5% of all dementias (Cairns et al., 2003). Neuropathologically, FTD is generally characterized by atrophy of the frontal and temporal lobes, which is apparent on macroscopic inspection (Cairns et al., 2007). FTD can also be divided into subtypes based on histological features of intracytoplasmic inclusions which are predominantly either tau-positive Pick bodies (Delacourte et al., 1996), tau-negative ubiquitin-positive inclusions including TDP-43 (Seelaar et al., 2007, Arai et al., 2006) and inclusions that are tau- and TDP-43 negative, but positive for ubiquitin and FUS (Neumann et al., 2009). Other subtypes based on features, such as those with p62-positive inclusions and those without significant histological features, are also occasionally encountered (Mackenzie et al., 2010). Cairns et al, 2003 charted the degree of atrophy and neuronal loss in these regions in FTD (Cairns et al., 2003). Due to the heterogeneity of neuropathological features of FTD, this study included cases from one subtype of FTD, which is characterized by the presence of ubiquitin-positive and tau-negative inclusions. Cortical thickness and neuronal density were reduced in FTD cases in the frontal, temporal and parietal cortices. Atrophy was hierarchical, with atrophy highest in frontal, followed by temporal, then parietal lobes. However, comparison with clinical information revealed heterogeneous clinical presentations, even in cases with similar pathological features.

Abnormal eating behaviors and corresponding obesity are frequently encountered in FTD patients (Ahmed et al., 2014), prompting the assessment of morphometry and pathology in regions of the hypothalamus known to be involved in appetite regulation in cases with FTD (Piguet et al., 2011). All cases had behavioral variant FTD but with two pathological subtypes, those with Pick body and those with TDP-43 inclusions. The hypothalami were subdissected from coronal tissue slabs and serially sectioned,

with sections taken at uniform intervals. The optical fractionator method was used to quantify cellular number on sections in the anterior and posterior regions. FTD cases were compared by their predominant pathology; those with TDP-43-predominant pathology had a significant loss of neurons in the posterior hypothalamus compared to controls and those with Pick body-predominant pathology. No changes in neuron number were found in the anterior hypothalamus across groups. Furthermore, no changes were found in neurons immunoreactive for proteins thought to be implicated in appetitive eating behaviors, such as orexin.

## 8. AIDS

AIDS is an acquired immunodeficiency virus that produces widespread and severe depletion of the CD4 antigen from lymphocytes, leading to compromised immune function and subsequent opportunistic infections (Dalglish et al., 1984). The AIDS virus may enter the cerebrum through circulation of infected macrophages (Gartner, 2000), or opportunistic infections that may compromise neurological functioning (Ketzler et al., 1990). A proportion of AIDS patients develop a dementia syndrome (Portegies, 1994). One study of 492 homosexual men in the USA showed that 15% had developed the AIDS dementia complex at the time of death (McArthur et al., 1993). The AIDS dementia complex is clinically marked by progressive dementia with cognitive components, such as executive deficits and learning dysfunction, as well as motor symptoms, including extrapyramidal symptoms and gait instability (Brew and Chan, 2014). AIDS dementia complex is a dementing disorder secondary to a viral infection, rather than a neurodegenerative dementia like AD or DLB. However, the disorder warrants inclusion in the present review as, despite the often severe clinical dysfunction, many AIDS cases show minimal histopathological changes to the brain at autopsy (Navia et al., 1986). Therefore, researchers using stereological methods have been able to make major advances in the understanding of the disorder by investigating subtle morphometric changes that occur in the absence of severe degenerative pathological lesions.

Three-dimensional designs using the Cavalieri method combined with point counting for the assessment of structural volume have revealed cerebral volume loss in AIDS cases, particularly in temporal and parietal regions, in the absence of changes to brain weight (Oster et al., 1993). The combination of volumetric analyses with optical density measures has also been employed in AIDS dementia complex research to estimate total neuronal number within structures. Two studies have shown no significant changes in neuron number in the CA1, CA2, CA3, CA4 and the *stratum granulare* of the dentate gyrus in the hippocampus in AIDS dementia complex (Korbo and West, 2000, Sa et al., 2000). However, reductions in CA3 and CA4 neuronal volumes, as well as total hippocampal volume, have been reported in AIDS cases; however, such changes are not related to clinical variables (Sa et al., 2000).

## 9. Conclusions

Stereological analysis has proved an important tool in the assessment of the major structural correlates of neurodegenerative dementing disorders. Despite limited sample size in many of the studies, largely due to the strict sampling protocol and labor- and time-consuming preparatory and analytical procedures, these studies have provided important mechanistic insights into the relationship between the pathological changes that define the clinical features in the dementias. Stereological procedures are particularly useful in dementing disorders that do not possess overt pathological lesions or histological features, such as AIDS dementia complex and certain subtypes of FTD (Cairns et al., 2007, Navia et al., 1986). Nevertheless, the studies reviewed here have, in large part, focussed on cellular morphology, specifically soma number or volume, with other structures, such as length of blood vessels and dendritic arbor, and volume of amyloid plaque pathology, meaning many stereological applications remain under-utilized. However, increased awareness of the importance and benefits of correct unbiased sampling and analytical protocol in a brain bank environment, as well as advances in analytical technology, may encourage future dementia researchers to incorporate novel stereological designs in pathology research.

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