What Counts in Brain Aging?
Design-Based Stereological Analysis of Cell Number

Jeffrey M. Long,[1] Peter R. Mouton,[2] Mathias Jucker,[3] and Donald K. Ingram[1]

[1]Molecular Physiology and Genetics Section, Gerontology Research Center, National Institute on Aging,
National Institutes of Health, Baltimore, Maryland.
[2]Department of Pathology and Neuropathology Laboratory, Johns Hopkins School of Medicine, Baltimore, Maryland.
[3]Department of Neuropathology, Institute of Pathology, University of Basel, Basel, Switzerland.

The advent and implementation of new design-based stereological techniques allows the quantification of cell number without the assumptions required when obtaining areal densities. These new techniques are rapidly becoming the standard for quantifying cell number, particularly in aging studies. Recently, studies using stereological techniques have failed to confirm earlier findings regarding age-associated neural loss. This newly emerging view of retained cell number during aging is having a major impact on biogerontology, prompting revaluation of long-standing hypotheses of age-related cell loss as causal for age-related impairments in brain functioning. Rather than focus on neuronal loss as the end-result of a negative cascade of neuronal injury, research has begun to consider that age-related behavioral declines may reflect neuronal dysfunction (e.g., synaptic or receptor loss, signal transduction deficits) instead of neuronal death. Here we discuss design-based stereology in the context of age-related change in brain cell number and its impact on consideration of structural change in brain aging. Emergence of this method of morphometrics, however, can have relevance to many areas of gerontological research.

S INCE the seminal work of Brody in the mid 1950s (1), the hypothesis that age-related cognitive changes result from loss of neurons and their interconnections, called synapses, has enjoyed much scientific and popular support. Evidence supporting the hypothesis has been derived from studies of humans as well as of many different species (1–8). In addition to reports of age-related decreases in neuron and synapses, other studies have reported age-related increases in the supporting cells of the brain, called glia (9–14). This increase in the number of glia includes microglia, which serve as brain macrophages, and astrocytes, which provide physical and trophic support for neurons. The idea that brain aging was associated with an increase in glia number and glia size (gliosis) coincident with a decrease in neurons fits well with the hypothesis that these events were somehow casually related. Until recently these observations concerning neuronal loss and gliosis were considered almost dogmatically and formed the basis for several hypotheses of brain aging, such as glucocorticoid stress (15,16), oxidative stress (17,18), inflammation (19), and apoptosis (20–22).

With the advent of new *design-based stereology*, however, controversy has emerged around·the major tenet of these hypotheses, leading to a reexamination of the question of age-related cell number changes in the brain. Utilizing these new counting techniques, several recent studies in a variety of species, including humans, have failed to confirm a global age-related loss in neuron number (23–27), even in subjects presenting age-related cognitive impairment (28,29). It is important for gerontologists to understand how this major conflict arose and to appreciate the implications of design-based stereology as a research tool for aging studies in general (30). To shed light on this issue, we will pursue three specific objectives in this review.

Within the context of age-related change in brain cell number, we first introduce the basic principles of design-based stereology. The goal is to provide insight on how these techniques result in more accurate and sometimes different estimates of cell number than previous methods. Importantly, the studies identified using nonstereological methods are not to be viewed as examples of poor research. Quite the contrary, they made use of the best quantitative methods available at that time. Second, we provide a brief review of recent studies in which design-based stereological methods were used to quantify age-related neuron loss and change in glia number. Third, we discuss the impact of these findings on several current hypotheses of brain aging. Although the focus of the review is on the nervous system, we also want to emphasize that design-based stereology can be applied to other areas of gerontological research. For a more detailed description of design-based stereological methods, the reader is directed to reviews by West (31) and Gundersen and colleagues (32). The application of these techniques to studies of neural systems has been reviewed recently by Geinsiman and colleagues (33), Coggeshall and Lekan (34), and Morrison and Hof (35).

DESIGN-BASED STEREOLOGY

The first design-based stereological technique, the disector principle, was introduced in 1984 (36), then expanded by Gundersen in subsequent papers into the optical disector and optical fractionator approaches (32,37–39). Design-based stereology can be used to produce accurate and unbiased estimates of several morphological parameters, such as volume, size, and length; however, the focus in this review will be on estimating the total number of cells in a defined brain region.

For investigators interested in estimating size (volume) parameters, several design-based and efficient estimators have

been developed in the past decade and half. Regional volume of tissue can be estimated using the point counting–Cavalieri combination (38), as recently demonstrated in a study comparing regional brain volumes in aging and Alzheimer's disease (40). Methods for estimating mean cell volume are based on the nucleator principle (32), as well as its more efficient derivative, the rotator method (41). Readers interested in a biological application of the rotator method to gerontological research are directed to a study of pigmented neurons in the human locus coeruleus (24).

For the purpose of this review, we will consider only four principles of stereology and only in reference to one type of stereological method, the *optical fractionator* (39). These four principles can be summarized as follows: (i) the use of systematic random sampling; (ii) the calculation of total number rather than densities; (iii) the counting of cells rather than cell profiles; and (iv) the partitioning of the variance to determine the sampling precision.

At the outset of this discussion, it should be emphasized that design-based stereology is really no more than a collection of methods used to *sample* biological tissue in an efficient and accurate manner. Indeed, stereological sampling principles are used in a wide array of nonbiological settings as well. Sampling is necessary when complete counts of objects within a structure are not practical, such as assessing the total number of neurons in large brain regions or cells in any other organ for that matter. In such instances, the next best approach is to acquire an efficient, precise, and accurate *estimate* of the total number. An accurate method is one without a systematic error (bias); thus as sampling increases, the estimate converges on the true value. Precision refers to the observed variation in individual estimates. Precision is increased by increasing the amount of sampling per subject (i.e., increasing the amount of work). Efficiency is defined as precision units divided by units of time (or unit of work). Stereological methods are designed to avoid systematic error and allow the experimenter to set the appropriate level of precision by controlling the intensity of sampling (42). The concepts of accuracy and precision are represented in Figure 1.
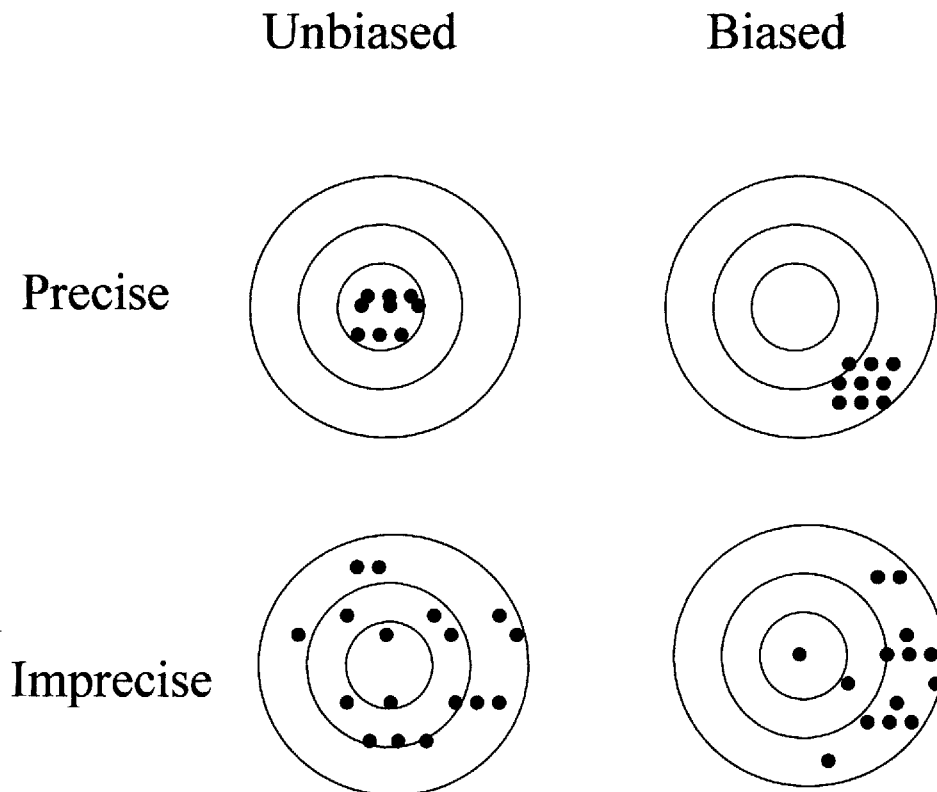

Figure 1. A pictorial representation comparing accuracy and precision. Black dots represent individual estimates of the true value, which in turn is represented as the center of the target. Both biased and unbiased methods can vary in their level of precision, depending upon the intensity of the sampling; the more of the region that is sampled, the more precise the estimate. However, biased methods introduce systematic error such that even high precision does not improve the accuracy of the estimate. Unbiased estimates, however, converge on the true value in response to increased sampling. Note that even with a relatively low level of sampling intensity, the average of the less precise unbiased estimates is the true value.

Systematic Random Sampling

Systematic random sampling allows for accurate sampling in a manner that is more efficient than either pure random or "representative" sampling (38). In the past many typical experiments involving cell counting would examine only a few representative sections of the brain region of interest. The assessment of cell number at representative sample locations (e.g., choosing only the best stained sections located at the mid region of the structure, or only counting cells in the dorsal part of the structure) contains the inherent assumption that the distribution of cells is the same in the nonsampled region of the structure as it is in the sampled region. The accuracy of the final estimate is influenced heavily by the accuracy of this assumption.

Both pure random sampling and systematic random sampling avoid the most severe assumptions of previous approaches by adhering to a simple principle, namely, ensure that each cell in the structure has an equal probability of being counted. Fulfilling this requirement ensures that the final estimate of cell number is a function of the total number of cells in the structure and is not disproportionally influenced by the number of cells in a particular subregion. It is therefore necessary that the entire structure of interest is available for assessment. There is no known method for determining the number of cells in missing tissue. In the absence of the complete structure, results can be reported only in terms of being representative of the missing tissue. Again, the validity of an estimate is directly related to the extent that this assumption is correct.

Although both systematic random and pure random sampling are unbiased, design-based stereology utilizes the former because it is more efficient. That is, systematic sampling captures more information about the number of cells in a structure per unit of work compared to random sampling (28). Accordingly, systematic sampling is used at every step of the process, from determining which sections in a series are sampled for counting, to which regions on each section are sampled, to the region in the thickness of the section where the cells are counted. In this way each cell has an equal probability of being sampled, hence the term "unbiased sampling."

Figure 2 presents this principle in practice. In this instance the brain structure of interest is the hippocampus of the mouse. As shown in Figure 2A, the first step of the sampling process involves making sections of the brain region containing the entire hippocampus. In the present example this results in approximately 60 equal sections (not all sections are represented in Figure 2A, and the hippocampus is not shown to scale). From these sections, taking sections separated by a constant spacing (i.e., every sixth) creates six separate subseries of sections, each with 10 sections (6 × 10 = 60 total sections). One subseries is chosen at random for analysis and is represented in Figure 2B. On each section of the subseries, a number of systematically distributed locations are selected for sampling. This sampling is accomplished by the random placement of a uniform grid over the section, and then sampling where line intersections fall over the region of interest. In Figure 2C "Xs" represent these line intersections. Finally, as shown in Figure 2D, at each location represented by an "X," a virtual three-dimensional probe is generated within the tissue. This virtual probe is called the optical disector as described below. The sampling intensity can be increased by either increasing the number of sections included in

each subseries (i.e., including every third section), by decreasing the spacing between sampling locations, or both. Note that at each level of sampling, the identification of regions to count is determined at random without regard to the underlying cells.

The superiority of systematic random sampling compared to the use of representative samples in studies of brain aging should be clearly evident. Indeed, unless the distribution of cells within a structure is homogeneous and, importantly, that this distribution does not change with age, the data collected on representative samples is suspect. With the benefit of hindsight, we should understand that many classic studies in brain aging assessed either cell number or cell morphology on a few representative sections. For example, in their reports of decreases in neuron cell number of human cortex, well-known studies by Brody (1) and Henderson and colleagues (4) counted cells on representative sections from the center of tissue blocks containing the regions of interest. Other examples include significant age-related decrease in neuron number (5) or increase in "summed gliosis" (but not glia density) (16) in the hippocampi of rats. In these studies brain cells were counted on representative sections from midpolar regions only. These data are methodologically biased because they rest on the assumption of a homogeneous distribution of cells. Furthermore, as described
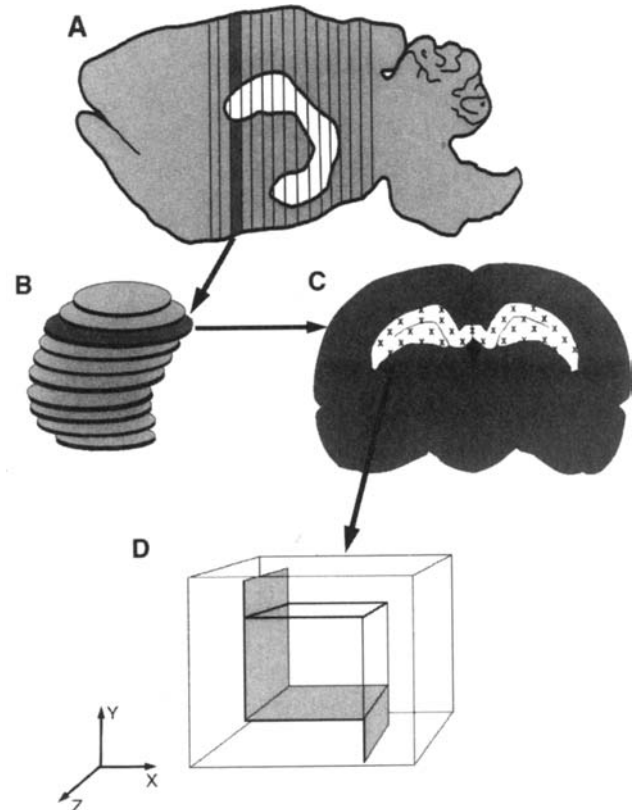

Figure 2. A pictorial representation of systematic random sampling within the fractionator sampling scheme. (A) The structure is completely sectioned. (B) A subseries is created via the selection of sections separated by a constant spacing. (C) On each section of the subseries, locations marked by a randomly superimposed grid are assessed for cells within a (D) three-dimensional probe placed within the thickness of the tissue. See text for details.

next, the reliability of these studies is based on assumptions inherent to cell densities and cell profile counting, rather than total number of cells.

Calculation of Total Number Rather Than Densities

Previous to the introduction of design-based stereology, the conventional method for reporting cell number was the calculation of areal densities (i.e., cells/mm^2) within a tissue section. This calculation is problematic, however, as differences in areal density measurements can reflect a change in either the numerator (number of cells) or the denominator (the size of the brain region). Emphasizing changes in the number of cells while discounting the possibility of changes in the region of interest introduces a severe methodological bias into density estimates. Figure 3 presents a representation of tissue processing artifacts that can occur in neural tissue. Investigators might be unaware of the amount of shrinkage that affects neural tissue during routine tissue processing. Shrinkage of frozen tissue up to 60% is common as a result of water exudation from the extracellular space of tissue during processing.

Haug (43,44) made the very significant observation that brain tissue from younger subjects shrinks substantially more than tissue from older subjects. This differential shrinkage artifact has important consequences for estimates of cell number based on areal density measurements. The left panel of Figure 3 represents tissue from an aged brain, the right panel from a young brain; both areas contain the same number of cells. For any constant area (represented by the superimposed box) the

areal density for the old brain is less than for the young, leading to the incorrect conclusion that cell number decreases with age.

Stereological methods avoid this shrinkage artifact by estimating the total number of cells in the entire region, not the cellular density within a circumscribed area. Importantly most of the published data, until recently, comparing cell number in subjects of different ages were based on estimates of areal densities rather than total number. This point should not be taken lightly. It means that all studies of brain aging that express age-related differences in cell number as areal densities (i.e., cells/mm^2) are methodologically biased. Unless considerable effort is used to show that the reference space remains constant, estimates of cell loss based on areal density estimates are suspect. These observations may explain why studies using design-based stereology have not confirmed widespread age-related neuron loss in human hippocampus (23), neocortex (26), and locus coeruleus (24,25) as reported by studies of cell densities (1,2,5).

In addition to biases from representative sampling and reporting densities, a third potential bias is assessment of cellular profiles rather than of cellular number. This principle is described in the next section.

Counting of Cells Rather Than Cell Profiles

Prior to the introduction of stereological methods, the standard method for identifying cells for quantification was based on the presence of cellular profiles. A cellular profile is the two-dimensional area of a cell that appears on the surface of a cut

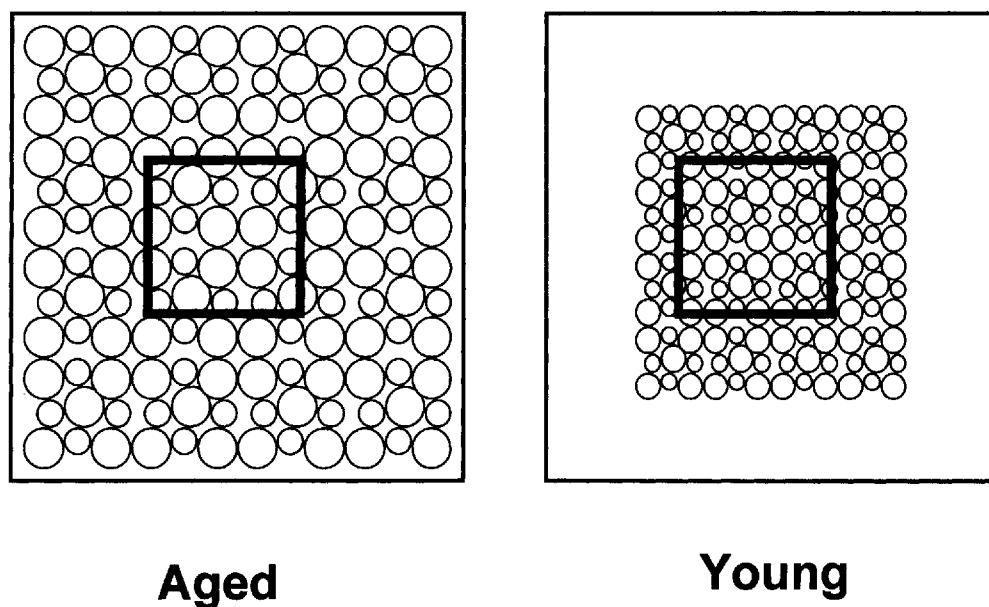**Aged**   **Young**

Figure 3. Representation of differential shrinkage observed in neural tissue from aged compared to young subjects. Either change in cell number and/or changes in region volume will affect any assessment of cell number expressed as an areal density (i.e., cells/mm^2). Thus, in the present example, even though the total numbers of cells are the same in the two panels, the areal densities within the superimposed box are different. Stereological methods avoid this shrinkage bias by estimating the total number of cells in the entire region, not within a circumscribed area.

section. The size, shape, and orientation of the cell relative to the plane of the knife affect the probability that any particular profile will appear. Two simple examples of this can be seen in Figure 4. As can be observed, the size and shape of the cells affect the probability of observing a profile. This means the number of cell profiles observed on a section is not necessarily related to the number of cells in the structure.

Researchers have been aware of this methodological issue for quite some time, and a number of attempts have been made to develop correction formulas. However, all correction factors require further assumptions regarding the size and shape of cells. For example, many correction factors assume that cells are round and of approximately equal and unchanging diameter. To the extent the assumptions are met, the correction factors work quite well. To the extent the assumptions are not met, the estimates are biased. Prior to the advent of design-based meth-

ods to count cells rather than profiles (see below), many studies reported age-related changes in cell number using assumption-based correction factors. For example, studies examining age-related hippocampal neuronal cell loss in humans (45,46) and rats (47,48) used the correction factor of Ambercrombie (49). However, it is known that the assumptions of spherically and constant diameter are not valid in these cases. Again, to the extent the assumptions of the correction factor were not met, the estimates are biased. Previous studies not using correction factors assumed that the number of profiles observed on a cut section was equal to the number of cells, an assumption that is likely untrue (34).

Design-based stereological methods solve the problem of profile counting without the assumptions inherent to correction factors. In its most efficient form, the technique is called the *optical disector*. The approach involves counting whole cells (not
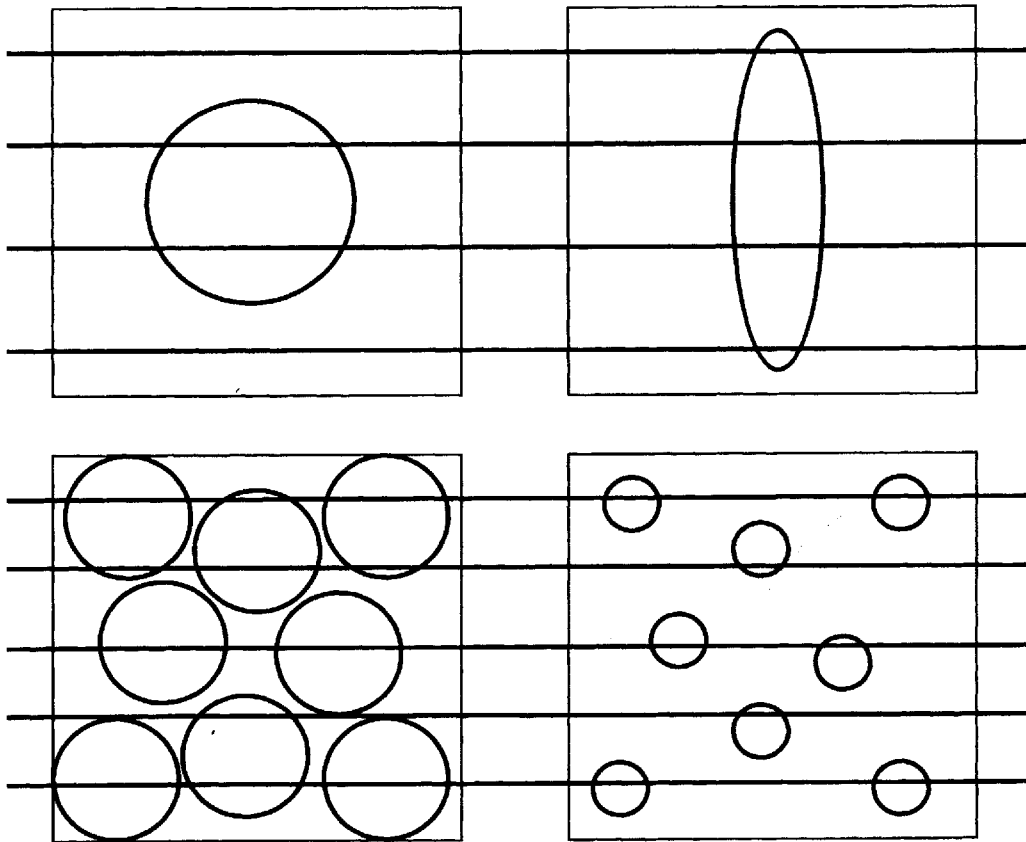

Figure 4. Examples of how the size and shape (relative to the sectioning plane) of cells can influence the probability of observing their profile on a cut section. The objects within the panels represent cells; the horizontal lines represent location of knife cuts. The cells in the two upper panels differ only in shape and the cells in the two lower panels differ only in size. Stereological methods bypass this bias by counting whole cells rather than cellular profiles.

profiles) within a three-dimensional probe called the optical disector (Figure 2D). An optical disector is a virtual stack of focal planes as viewed along the z-axis of a relatively thick section of tissue (16–30 μm). In practice, this is accomplished by moving the focal plane of a high-power objective from the top to the bottom of the section (e.g., moving through the z-axis) and identifying and counting the leading edge of each cell. Cells are counted according to unbiased counting rules, that is, cells touching either left or bottom sides of the counting frame, and the bottom plane of the disector, are excluded. These counting rules ensure that all cells have an equal probability of being sampled, regardless of size, shape, or orientation (36). Each cell is counted only once, and only when its leading edge (top or bottom) falls within the volume of the disector, with no further assumptions.

Prior to the advent of design-based stereological approaches, studies of brain aging quantified cell number by counting cellular profiles (either with or without the use of correction factors; correction factors in which the assumptions either were or were not met). Thus, any age-related differences in size, shape, and orientation of the cells confound the data and may raise questions about the conclusions of these studies.

Outline of the Optical Fractionator

The three principles discussed above provide a basis for understanding the stereological counting method referred to as the optical fractionator. This technique combines the optical disector and the fractionator sampling scheme. The fractionator sampling scheme provides the estimate of total number as the sum of objects counted, multiplied by the reciprocal of the fraction of the brain region sampled. The sampling scheme is unbiased because the selection of which sections to sample and the locations sampled on each section are determined via systematic random sampling (Figure 2).

The systematic nature of the sampling allows for easy calculation to determine the fraction of tissue in which cells were counted. For example, if complete sectioning of a structure results in 60 sections, and every 6th section is sampled (for a total of 10 sections), then the first sampling fraction would be 1/6 (Figure 2B; this is called the section sampling fraction or *ssf*). If every possible location on each section is not sampled, but only systematically distributed locations and the sum area of these locations equals 1/100 of the tissue, then 1/100 is the value of the second sampling fraction (Figure 2C; this is called the area sampling fraction or *asf*). The third sampling fraction reflects that cells are not counted in the entire thickness of the tissue at each sampling location. Instead, a three-dimensional probe (optical disector) of a known height is placed in the tissue (Figure 2D). The thickness of the tissue divided by the height of the disector is the third sampling fraction. (This is called the tissue sampling fraction or *tsf*.) The estimate of total cell number is therefore the sum of cells counted (ΣQ^-), multiplied by the reciprocal of the three fractions of the brain region sampled as represented by the equation:

$$N = \Sigma \, Q^- \, (1/ssf) \, (1/asf) \, (tsf)$$

Where N is the estimate of the total cell number and $\Sigma \, Q^-$ is the number of counted cells on all sections. The reader is directed to West and colleagues (39) for a detailed description of the optical fractionator.

Partitioning of the Variance to Determine Sampling Precision

Design-based stereology is a mathematically unbiased approach; thus, as the sampling intensity within each brain increases, the estimates of total cell number converge on the true value (Figure 1). Comparison of pilot experiments in which the sampling is increased systematically allows one to determine the optimal sampling intensity to capture the majority of within-sample variation in cell number. For results obtained using methodological unbiased methods, variation that is not the result of within-sample variation is by definition the result of biological variation. Thus, knowing the within-sample variance and total group variance makes it possible to determine the percentage of the total variance attributable to variation in the individual estimates (method error) and the percentage attributable to true biological variability among the subjects (i.e., the true difference in number of cells for different subjects). This important feature of design-based methods permits an efficient sampling scheme to allocate resources to the factor that contributes the greatest percentage of variation to the total variance. For instance, if high variability in individual estimates contribute most to the total variance, then the sampling intensity within each subject should be increased. However, if biological variability among subjects is the major source of the total variance, then it is more efficient to increase the number of subjects in the study as opposed to sampling more within the existing number of subjects. As a general rule, it is efficient to set the level of sampling precision such that the biological variability contributes at least half of the total variance. Partitioning the total variance into its methodological and biological components is an important feature of the design-based stereology approaches. The reader is directed to West (31) for further details.

This issue is important because of the substantial variation in brain cell number among subjects from the same age category. Many of the early studies of brain aging examined too few subjects to assess this high level of normal variation accurately and too few subjects to make appropriate use of inferential statistics. This issue is especially problematic in studies of neurodegenerative diseases in elderly humans, as it is often difficult to acquire a sufficient number of samples necessary to capture the observed biological variance in this population (50,51). Nevertheless, to generalize the results from the subjects in a specific study to the corresponding population from which they were sampled, it is necessary to have enough subjects to capture the majority of biological variability within that population. Importantly, it is the mathematically unbiased nature of design-based stereology that allows the sampling precision to be calculated. Previous assumption-based methods (i) did not utilize random sampling, (ii) did not assess total number, and (iii) identified cells rather than profiles, and thus are mathematically biased. Because these approaches could not account for all sources of variation in cell number, they do not allow the variance to be partitioned for the purpose of optimizing the sampling precision.

Summary of Design-Based Stereological Methods

The four principles of design-based stereology described above (in conjunction with stereological tenets not addressed) result in more accurate, precise, and efficient sampling designs of biological tissue than are possible with nonstereological methods. Stereological approaches avoid the assumptions related to

areal densities, correction factors, and shrinkage artifacts. Data obtained prior to the era of design-based stereological methods are open to question because, in general, it is unknown whether the inherent assumptions in the quantitative collection methods were met, as explained in the following example.

If a study quantifies age-related changes in number were based upon profile counts in a circumscribed brain region on representative sections, then the assumptions include: (i) cells are round and approximately the same size; (ii) no differential processing artifacts between old and young tissue; and (iii) there is a homogeneous distribution of the cells within the structure. If a correction factor is applied to the data, this adds another assumption. Many nonstereological studies may include only two of these assumptions, some perhaps only one. To repeat, provided the assumptions are met, the data are not biased. The advantage of design-based stereological methods is that these and other limitations are avoided, as is the need to confirm the validity of assumptions. When there are discrepancies between data from nonstereological methods and data collected by design-based stereological methods, the parsimonious conclusion is that at least one or more of the assumptions in the nonstereological method were not met.

The reader who is interested in comparing stereological and nonstereological quantitative studies is directed to Tables 1and 2 of an excellent review by Geinisman and colleagues (33). This review lists studies examining age-related changes in neuron and synapse number in the hippocampus of different species, including the major finding of each study, the counting methods used, any correction factors, and so forth. It is safe to state that prior to the introduction of the disector principle in 1984, studies of cell number calculated the areal densities of profiles, and thus, their results are suspect due to the reasons described above. Although this does not mean that these data are necessarily incorrect (the inherent assumptions may have been met), it does mean that the issue of age-related change in cell number in these studies has to be reexamined with design-based stereological methods to confirm or question the earlier data.

In the previous sections we have outlined the advantages of using design-based stereological quantification techniques. In the remaining sections we turn our attention to newly emerging stereological data as they pertain to age-related change in brain cell number. For the reasons outlined above, the reader is cautioned about comparing estimates based on volume densities (cell/mm^3) with previous studies of areal densities (cell/mm^2). It is unclear how data from design-based stereological and nonstereological studies compare (34). Thus, whereas comparisons of absolute numbers can be assessed among design-based studies, no direct comparison of quantitative values between stereological and nonstereological studies will be attempted.

REVIEW OF RECENT STEREOLOGICAL FINDINGS

Table 1 presents some of the recent findings from studies utilizing design-based stereological methods to assess age-related changes in neural parameters. What is striking is the number of studies reporting no significant age-related change in brain cell number in a variety of species. An earlier example is the demonstration first in 1994 (24), then again in 1997 (25), that cell number in the human locus coeruleus is stable across the adult life span, in contrast to earlier reports of substantial cell loss with age (2).

Table 1. Results of Design-Based Stereological Quantitation of Cell/Synapse Number in Aging

Species	Cell Type	Brain Region	Result	Reference
Human	Neuron	Cortex	→	26
Human	Neuron	Locus coeruleus	→	24
			→	25
Human	Neuron	Hippocampus		
		CA1	→ ↓	52
				53
		CA2/3	→	52
			→ ↓	53
		Hilus	→	52
			→ ↓	53
		Subiculum	↓	52
			↓	53
Human	Synapse	CA1		59
Monkey	Neuron	Striate cortex	→	80
Monkey	Neuron	Entorhinal cortex	→	81
Rat	Neuron	Hippocampus	→	29
			→	28
Rat	Astrocyte	Dentate gyrus		54
Rat	Astrocyte	CA1	→ ↓ ↑	55
Rat	Synapse	Dentate gyrus		57
Rat	Synapse	CA1	→	58
Mouse	Neuron	Hippocampus	→	27
Mouse	Astrocyte	Hippocampus	→	82
Mouse	Microglia	Hippocampus	→	82
Mouse	Synaptic bouton	Hippocampus	→	27

Note: → = No statistically significant change with increasing age.

Less concordant are the studies of neuron number in the human hippocampus during aging. To date, two published studies have quantified neuron number in different subregions of the human hippocampal formation. Although both agree that age-related loss of neuron number occurs in the subiculum but not in the CA2/3 regions of the hippocampus, these reports differ in their assessment of two other hippocampal subregions. The study of West and colleagues (52) reports age-related loss of neurons in the hilus but not the CA1 region, whereas the study by Simic and colleagues (53) finds the opposite pattern.

An obvious question is how can two studies utilizing theoretically unbiased stereology procedures show wide disparities in the age-related pattern of neuronal loss in the human hippocampus? Simic and colleagues (53) propose one possible explanation. These authors suggest that the number of subjects in the two studies was not sufficient to reduce the standard error to a point where the effects of aging could be detected reliably. Thus, these conflicting data address an issue familiar to gerontologists; namely, that increased variability (on many measures) observed in aged populations necessitates relatively large sample sizes to capture the expected high level of biological variability.

As shown in Table 1, the cumulative data from design-based studies suggest no change in cell number during aging. A second age-related change reported is an increase in astrocyte number in the dentate gyrus of the hippocampus in rats (54). Although statistically significant, this increase is minor, less than 20% from 3–25 months of age. However, two independent studies found no evidence for age-related hippocampal neuronal loss in rats, even in rats exhibiting memory deficits

(28,29). Furthermore, two other studies did not find a statistically significant increase in the number of hippocampal astrocytes in either rats (55) or mice (56).

To date, only a few reports have examined age-related changes in synapse number utilizing design-based stereological methods. In rats, an early study found an age-related decrease in the ratio of axospnious synapses per neuron in the dentate gyrus region of the hippocampus (57). A preliminary report in rats found no significant age-related change in the CA1 region of the hippocampus (58). In mice, Calhoun and collaborators (27) reported a significant correlation between the number of synaptophysin-positive synaptic boutons in the dentate gyrus and in performance on a test of spatial memory. Although no age-related decline in synaptic bouton number was found in this study, this correlation suggests that synapse loss is related to deficits in spatial memory. In humans, results from an early report suggest the density of synapses in the CA1 region declines with age (59). In brief, early data suggest different species and brain regions may have different patterns of age-related changes in synapse number. The accurate estimation of synapse number is an exciting area for future design-based neuromorphometric research.

Although the present review focuses on cell numbers, the reader should be aware that other parameters (e.g., volume) can be useful when studying age-related brain change. In support of this view, recent evidence from a study using design-based stereological methods shows that humans with Alzheimer's disease show a strong correlation between cognitive performance and total cortical volume (40).

IMPACT ON THEORIES OF BRAIN AGING

Although design-based stereology represents a relatively new area in brain aging research, the results from studies using this approach generally do not support the hypothesis that age-related cognitive changes result from a global loss of neurons. This view suggests that factors other than simple neuron loss underlie age-related cognitive decline. As mentioned above, there is evidence to support the hypothesis that cognitive performance is related to either the number of synapses (27) or their plasticity (60). Furthermore, the age-related loss of white matter (myelin) in primates has been proposed as the basis for age-related cognitive decline (61). These studies illustrate that perturbations in cell structure during aging may have important implications for the functional integrity of the cell. Because stereology assesses morphology only, physiological or biochemical experiments are required to further investigate mechanisms of aging on cell function. For example, recent evidence suggests that dysfunctional calcium processing by neurons contributes to age-related learning impairments (62,63).

Several hypotheses of brain aging point to age-related neuronal loss as their central tenet. Among these are glucocorticoid stress, oxidative stress, apoptosis, and inflammation theories of brain aging. The glucocorticoid stress model posits that glucocorticoids accelerate hippocampal aging by either intrinsically killing hippocampal neurons (which contain receptors for these hormones), or by making them more vulnerable to neurological insults (64,65). Prolonged overexposure to glucocorticoids is hypothesized to exacerbate small neural perturbations in the aged brain that have no deleterious effect on younger brains. In rats, research utilizing nonstereological methods supports the

relationship between aging, glucocorticoid level, and neuronal loss in the CA1 region of the hippocampus (see reviews in 15,64). Moreover, it was reported recently that cortisol levels in elderly humans correlate with hippocampal atrophy (as measured via magnetic resonance imaging) and memory (66).

The oxidative stress hypothesis of aging posits that age-related loss of function is a result of the progressive accumulation of molecular oxidative damage via an array of reactive oxygen metabolites or free radicals (see references 17,18 for review). These reactive oxygen species cause modification of DNA, loss of sulfhydryls in proteins, and peroxidation of membrane fatty acid chains. Overall, reactive oxygen metabolites damage macromolecules and can lead to cell death. Considerable evidence exists implicating the involvement of oxidative stress in neuronal loss associated with stroke and Alzheimer's disease (67).

A consideration of programmed cell death (apoptosis) provides a mechanistic corollary to the above two hypotheses. Glucocorticoids (21) and free radicals (68) have been shown to stimulate apoptotic pathways. Whatever the stimulatory factors involved in apoptosis, research interest in this hypothesis is clearly based on documenting cell loss. Investigation of apoptotic mechanisms has major implications for understanding effects of aging in many organ systems (20,22), including brain. As one example, there has been interest in apoptotic mechanisms related to possible neurotoxic effects of dopamine metabolism. Dopamine has been shown to autoxidize, thus forming free radicals (69); in vitro, mouse thymocytes undergo apoptosis in response to dopamine (70). Thus, normal dopamine activity may over time induce apoptosis of striatal dopaminergic neurons. Using areal density estimates, Zhang and colleagues (71) reported an age-related increase in the number of striatal cells, stained using a TUNEL technique for identifying apoptotic cells. In vitro (72) and in vivo (73) application of dopamine to striatal cells resulted in TUNEL-labeled cells, likely acting through the dopamine D_2 receptor.

The inflammation hypothesis posits that over time, small cumulative insults perturb the central nervous system, resulting in low-level gliosis and inflammation (19). The magnitude of the microglia and astrocytic response to subsequent neural trauma (i.e., concussions, small infarcts, and systemic infections resulting in fever) may be increased due to the brain's chronic state of low-level gliosis. This increased inflammatory response by glia could make the aged brain more susceptible to neurodegeneration. A major tenet of this hypothesis is whether there is an age- or disease-associated proliferation of glia or whether there is a change in the reactive state of these cells as evidenced by their altered morphology.

Senescent processes are certainly multifactorial; thus, each of the hypotheses discussed above is useful for focusing on specific mechanisms. Whether the mechanism is glucocorticoids or oxidative stress, programmed cell death, or hyperinflammatory responses, a common feature in each of the hypotheses is an age-related change in a neural parameter and, most critically, neuronal loss. What then is the consequence of the newly emerging view of retained neuron number in aging for these theories? Clearly if this view is bolstered by future studies, then hypotheses of brain aging must be modified to fit the empirical data. Rather than focus on neuronal loss as the end-result of a negative cascade of neuronal injury, research could begin to

consider that age-related behavioral declines may reflect neuronal dysfunction (e.g., synaptic or receptor loss, signal transduction deficits, etc.) instead of neuronal death.

It is important to make the distinction between age-related cell loss and cell death. Several recent papers have provided evidence that neurogenesis occurs in the hippocampus of adult rodents, monkeys, and humans (74–76). If this phenomenon occurs in the absence of neuronal death, the result would be the appearance of an age-related increase in neuron number. Although it has been shown that neurogenesis in mice decreases with age (77), the total number of murine hippocampal neurons appears to remain constant across the life span (27). Thus, across the life span there might exist an ongoing balance of neurogenesis and neuronal death that would maintain the total number of neurons relatively constant.

Reestablishing appropriate synaptic connections during neuronal turnover is surely important in maintaining the neurobiological substrates of memory. Although neuronal loss may not be a hallmark in aging, perhaps synaptic disconnectivity is the vital factor. Indeed, the implication of a recent stereological analysis of hippocampal volume in behavioral characterized rats is that the effects of aging are regionally and circuit specific with the suggestion of a connectional reorganization as a more important mechanism than cell loss (78). In addition, recent evidence suggest that synaptic and axonal aberrations in relation to amyloid plaques may contribute to deficits observed in age-related diseases such as Alzheimer's disease (79).

Conclusion

Age-related neuromorphometric data are important to support many hypotheses in gerontology. Because empirical evidence continually modifies and suggests new hypotheses of senescence, it is imperative that these hypotheses are based on reliable morphometric data. We have outlined the new design-based stereological methods to show why these techniques generate accurate and precise estimates of total number of cells in defined brain regions. It should be clear that this approach has wide potential beyond morphological analysis of brain tissue. Stereological analysis of any structural parameter in any tissue is possible in principle, and should be considered important for accurate confirmation of any hypothesis in gerontology. Therefore, we anticipate that gerontologists will begin to appreciate the advantages of design-based stereology and the biases associated with nonstereological quantification methods in designing and evaluating future experiments involving cell or synapse counting.

The implementation of design-based stereology is relatively easy for most tissue and most cell types. With a little forethought, sectioning tissue by utilizing systematic random sampling requires no more effort than conventional sectioning methods. Moreover, several commercial companies sell computer-based systems that interface with most conventional microscopes and perform many of the tedious and repetitive tasks associated with design-based stereology (i.e., determining the random placement of disector locations, keeping track of the counts on each section, calculating the final value, etc.). Companies selling stand-alone stereology systems are listed in the appendix. Thus, in principle as well as in practice, design-based stereology will likely occupy an increasing greater role in gerontological research because it counts.

REFERENCES

1. Brody H. Organization of the cerebral cortex III. *J Comp Neurol.* 1955; 102:511–556.
2. Vijayashankar N, Brody H. A quantitative study of the pigmented neurons in the nuclei locus coeruleus and subcoeruleus in man as related to aging. *J Comp Neurol.* 1979;38:490–497.
3. Ordy JM, Brizzee KR, Kaack B, Hansche J. Age differences in short-term memory and cell loss in the cortex of the rat. *Gerontology.* 1978;24: 276–285.
4. Henderson G, Tomlinson BE, Gibson PH. Cell counts in human cerebral cortex in normal adults throughout life using an image analyzing computer. *J Neurol Sci.* 1980;46:113–136.
5. Landfield PW, Braun LD, Pitler TA, Lindsey JD, Lynch G. Hippocampal aging in rats: a morphometric study of multiple variables in semithin sections. *Neurobiol Aging.* 1981;2:265–275.
6. Mani RB, Lohr JB, Jeste DV. Hippocampal pyramidal cells and aging in the human: a quantitative study of neuronal loss in sectors CA1 to CA4. *Exp Neurol.* 1986;94:29–40.
7. Flood DG, Coleman PD. Neuron numbers and sizes in aging brain: comparisons of human, monkey, and rodent data. *Neuro Biol Aging.* 1988;9: 53–463.
8. Issa AM, Rowe W, Gauthier S, Meaney MJ. Hypothalamic-pituitary-adrenal activity in aged, cognitively impaired and cognitively unimpaired rats. *J Neurosci.* 1990;10:3247–3254.
9. Hansen LA, Armstrong DM, Terry RD. An innumohistochemical quantification of fibrous astrocytes in the aging human cerebral cortex. *Neurobiol Aging.* 1987;8:1–6.
10. Sturrock RR. A quantitative lifespan study of changes in cell number, cell division and cell death in various regions of the mouse forebrain. *Neuropathol Appl Neurobiol.* 1979;5:433–456.
11. Sturrock RR. A comparative quantitative and morphological study of aging in the mouse neostriatum, indusium griseum and anterior commissure. *Neuropathol Appl Neurobiol.* 1980;6:51–68.
12. Mandybur TI, Ormsby I, Zemlan FP. Cerebral aging: a quantitative study of gliosis in old nude mice. *Acta Neuropathol.* 1989;77:507–513.
13. Bronson RT, Lipman RD, Harrison DE. Age-related gliosis in the white matter of mice. *Brain Res.* 1993;58:124–128.
14. Sheng JG, Mrak RE, Griffin WST. Enlarged and phagocytic, but not primed, interleukin-1α-immunoreactive microglia increase with age in normal human brain. *Acta Neuropathol.* 1998;95:229–234.
15. Sapolsky RM. *Stress, the Aging Brain, and the Mechanisms of Neuron Death.* Cambridge, MA: The MIT Press; 1992.
16. Landfield PW, Waymire JC, and Lynch G. Hippocampal aging and adrencorticoids: quantitative correlations. *Science.* 1978;202,1098–1102.
17. Harman D. Free-radical theory of aging. Increasing the functional life span. *Ann NY Acad Sci.* 1994;717:1–15.
18. Sohal RS, Weindruch R. Oxidative stress, caloric restriction and aging. *Science.* 1996;273:59–63.
19. McGeer PL, McGeer EG. The inflammatory response system of brain: implicatons for therapy of Alzheimer and other neurodegenerative diseases. *Brain Res Rev.* 1995;21:195–218.
20. Zakeri Z, Lockshin RA. Physiological cell death during development and its relationship to aging. *Ann NY Acad Sci.* 1994;719:212–229.
21. Reagan LP, McEwen BS. Controversies surrounding glucocorticoid-mediated cell death in the hipppcampus. *J Chem Neuroanat.* 1997;13: 149–167.
22. Warner HR, Hodes RJ, Pocinki K. What does cell death have to do with aging? *J Am Geriatr Soc.* 1997;45:1140–1146.

23. West MJ. Regionally specific loss of neurons in the aging human hippocampus. *Neurobiol Aging.* 1993;14:287–293.

24. Mouton PR, Pakkenberg B, Gundersen HJG, Price DL. Absolute number and size of pigmented locus coeruleus neurons in young and aged individuals. *J Chem Neuroanat.* 1994;7:185–190.

25. Ohm TG, Busch C, Bohl J. Unbiased estimation of neuronal numbers in the human nucleus coeruleus during aging. *Neurobiol Aging.* 1997; 18:393–399.

26. Pakkenberg B, Gundersen HJG. Neocortical neuron number in humans: effect of sex and age. *J Comp Neurol.* 31997;84:312–320.

27. Calhoun ME, Kurth D, Phinney AL, et al. Hippocampal neuron and synaptophysin-positive bouton number in aging C57BL/6 mice. *Neurobiol Aging.* 1998;19:599–606.

28. Rapp PR, Gallagher M. Preserved neuron number in the hippocampus of aged rats with spatial learning deficits. *Proc Natl Acad Sci USA.* 1996; 93:9926–9930.

29. Rasmussen T, Schliemann T, Sorensen JC, Zimmer J, West MJ. Memory impaired aged rats: no loss of principal hippocampal and subicular neurons. *Neurobiol Aging.* 1996;17143–147.

30. Wickelgren I. Is hippocampal cell death a myth? *Science.* 1996;271: 1229–1230.

31. West MJ. New stereological methods for counting neurons. *Neurobiol Aging.* 1993;14:275–285.

32. Gundersen HJG, Bagger P, Bendtsen TF, et al. The new stereological tools: disectors, fractionator, nucleator and point sampled intercepts and their used in pathological research and diagnosis. *APMIS.* 1988;96: 875–881.

33. Geinisman Y, de Toledo-Morrell L, Morrell F, Heller RE. Hippocampal markers of age-related memory dysfunction: behavioral, electrophysiological and morphological perspectives. *Prog Neurobiol.* 1995;45:223–252.

34. Coggeshall RE, Lekan HA. Methods for determining numbers of cells and synapses: a case for more uniform standards of review. *J Comp Neurol.* 1996;364:6–15.

35. Morrison JH, Hof PR. Life and death of neurons in the aging brain. *Science.* 1997;278:412–419.

36. Sterio DC. The unbiased estimation of number and sizes of arbitrary particles using the disector. *J Microsci.* 1984;134:127–136.

37. Gundersen HJG. Stereology of arbitrary particles: a review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson. *J Microsc.* 1986;143:3–45.

38. Gundersen HJG, Jensen EB. The efficiency of systematic sampling in stereology and its perdiction. *J Mirosc.* 1987;147:229–263.

39. West MJ, Slomianka L, Gundersen HJG. Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *Anat Rec.* 1991;231: 482–497.

40. Mouton PR, Martin LJ, Calhoun ME, Palforms G, Price DL. Cognitive decline strongly correlates with cortical atrophy in Alzheimer's dementia. *Neurobiol Aging.* 1998;19:371–377.

41. Jensen EB, Gundersen HJG. The rotator. *J Microsc.* 1993;170:35–44.

42. West MJ. Stereological methods for estimating the total number of neurons and synapses: issues of precision and bias. *TINS.* 1999;22:51–61.

43. Haug H, Kuhl S, Mecke E, Sass N-L, Wasner K. The significance of morphmetric procedures in the investigation of age changes in cytoarchitectonic structures of human brain. *J Hirnforschung.* 1984;25:353–374.

44. Haug H. History of neuromorphometry. *J Neurosci Methods.* 1986; 18:1–17.

45. Mouritzen Dam A. The density of neurons in the human hippocampus. *Neuropathol Appl Neurobiol.* 1979;5:249–264.

46. Bertoni-Freddari C, Fattoretti P, Casoli T, Meier-Ruge W, Ulrich J. Morphological adaptive response of the synaptic junctional zones in the human dentate gyrus during aging and Alzherimer's disease. *Brain Res.* 51990;17:69–75.

47. Knox CA. Effects of aging and chronic arterial hypertension the cell populations in the neocortex and archicortex of the rat. *Acta Neuropthol (Berl).* 1982;56:139–145.

48. Meaney MJ, Aitken DH, van Berkel C, Bhatnagar S, Sapolsky RM. Effect of neonatal handling on age-related impairments associated with the hippocampus. *Science.* 1998;239:766–768.

49. Ambercombie M. Estimation of nuclear populations from microtome sections. *Anat Rec.* 1946;94:239–247.

50. Rapp PR, Burwell RD, West MJ. Individual differences in aging: implications for stereological studies of neuron loss. *Neurobiol Aging.* 1996;17:495–496.

51. Ball MJ, West MJ. Aging in the human brain: a clarion call to stay the course. *Neurobiol Aging.* 1998;19:1.

52. West MJ, Coleman PD, Flood DG, Troncoso JC. Differences in the pattern of hippocampal neuronal loss in normal ageing and Alzheimer's disease. *Lancet.* 1994;344:769–772.

53. Simic G, Kostovic I, Winblad B, Bogdanovic N. Volume and number of neurons of the human hippocampal formation in normal aging and Alzheimer's disease. *J Comp Neurol.* 1997;379: 482–494.

54. Pilegaard K, Ladefoged O. Total number of astrocytes in the molecular layer of the dentate gyrus of rats at different ages. *Anat Quant Cyto Histol.* 1996;18:279–285.

55. Bhatnagar M, Cintra A, Chadi G, et al. Neurochemical changes in the hippocampus of the brown norway rat during aging. *Neuobiol Aging.* 1997;18:319–327.

56. Long JM, Kalehua AN, Muth NJ, et al. Stereological analysis of astrocyte and microglia in aging mouse hippocampus. *Neurobiol Aging.* 1998; 19:497–503.

57. Geinisman Y, de Toledo-Morrel L, Morrell F, Persina IS, Rossi M. Age-related loss of axopinous synapses formed by two afferent systems in the rat dentate gyrus as revealed by the unbiased stereological dissector technique. *Hippocampus.* 1992;2:437–444.

58. Sullivan P, Feng Z, Price DA, et al. Unbiased estimation of possible age-related changes in total number of synapses in the hippocampal CA1 region of the rat. *Soc Neurosci Abs.* 1997;23:576.2.

59. Hamrick JK, Sullivan PG, Scheff SW. Estimation of possible age-related changes in synaptic density in the hippocampal CA1 stratum radiatum. *Soc Neurosci Abs.* 1998;24:783.1.

60. Geinisman Y, Disterhoft JF, Gundersen HJG, et al. Trace eyeblink conditioning induces a restructuring of preexisting synapses rather than synaptogenesis. *Soc Neurosci Abs.* 1998;24:560.1.

61. Peters A, Rosene DL, Moss MB, et al. Neurobiological bases of age-related cognitive decline in the rhesus monkey. *J Neuorpathol Exp Neurol.* 1996;55:861–874.

62. Disterhoft JF, Thompson LT, Moyer JR, Mogul DJ. Calcium-dependent afterhyperpolarization and learning in young and aging hippocampus. *Life Sci.* 1996;59:413–420.

63. Thibault O, Landfield PW. Increase in single L-type calcium changes in hippocampal neurons during aging. *Science.* 1996;272:1017–1020.

64. Landfield PW. Nathan Shock Memorial Lecture 1990. The role of glucocorticoids in brain aging and Alzheimer's disease: an integrative physiological hypothesis. *Exp Gerontol.* 1994;29:3–11.

65. Sapolsky RM, Krey LC, McEwen BS. Prolonged glucocorticoid exposure reduces hippocampal neuron number: implication for aging. *J Neurosci.* 1985;5:1222–1227.

66. Lupien SJ, de Leon M, de Santi S, et al. Cortisol levels during human aging predict hippocampal atrophy and memory deficits. *Nat Neurosci.* 1998;1:69–73.

67. Beal MF. Mitochondria, free radicals and neurodegeneration. *Curr Opin Neurobiol.* 1996;6:661–666.

68. Papa S, Skulachev VP. Reactive oxygen species, mitochondria, apoptosis and aging. *Mol Cell Biochem.* 1997;174:305–319.

69. Grimes JD, Hassan MN, Thakar J. Antioxidant therapy in Parkinson's disease. *Can J Neurol Sci.* 1987;14:483–487.

70. Offen D, Ziv I, Gorodin S, Barzilai A, Malik Z, Melamed E. Dopamine-induced programmed cell death in mouse thymocytes. *Biochim Biophys Acta.* 1995;1268:171–177.

71. Zhang L, Kokkonen G, Roth GS. Identification of neuronal programmed cell death in situ in the striatum of normal adult rat brain and its relationship to neuronal death during aging. *Brain Res.* 1995;677:177–179.

72. Shinkai T, Zhang L, Mathias SA, Roth GS. Dopamine induces apoptosis in cultured rat striatal neurons; possible mechanism of D2-dopamine receptor neuron loss during aging. *J Neurosci Res.* 1977;47:393–399.

73. Hattori A, Lou Y, Umegaki H, Munoz J, Roth GS. Intrastriatal injection of dopamine results in DNA damage and apoptosis in rats. *Neuroreport.* 1998;9:2569–2572.

74. Eriksson, PS, Perfilieva E, Bjork-Eriksson T, et al. Neurogenesis in the adult human hippocampus. *Nature Med.* 1998;4:1313–1317.

75. Gould E, Tanapat P, McEwen BS, Flugge G, Fuchs E. Proliferation of

granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. *Proc Natl Acad Sci USA.* 1998;95:3168–3171.

76. Kempermann G, Kuhn HG, Gage FH. More hippocampal neurons in adult mice living in an enriched environment. *Nature.* 1997;386:493–495.

77. Kempermann G, Kuhn HG, Gage FH. Experienced-induced neurogenesis in the senescent dentate gyrus. *J Neurosci.* 1998;18:3206–3212.

78. Rapp PR, Stack EC, Gallagher M. Morphometric studies of the aged hippocampus: I. Volumetric analysis in behaviorally characterized rats. *J Comp Neurol.* 1999;403:459–470.

79. Phinney A, Deller T, Stalder M, et al. Axonal aberration and dyssynaptogenesis in relation to amyloid plaque formation in APP transgenic mice. Submitted.

80. Kim CBY, Pier LP, Spear PD. Effects of aging on numbers and sizes of neurons in histochemically defined subregions of monkey striate cortex. *Anat Rec.* 1997;247:119–128.

81. Gazzaley AH, Thakker MM, Hof PR, Morrison JH. Preserved number of entorhinal cortex layer II neurons in aged macaque monkeys. *Neurobiol Aging.* 1977;18:549–553.

82. Long JM, Kalehua AN, Muth NJ, et al. Stereological estimation of total microglia number in mouse hippocampus. *J Neurosci Methods.* 1998;84: 101–108.

Appendix

C.A.S.T. Olympus of Danmark A/S—E-mail: mikro@olympus.dk

MicroBrightField, Inc.—http://www.microbrightfield.com

NeuroZoom—http://www-hbp-np.scripps.edu/ServerData/HTMLs/Software/Software.html

System Planning and Analysis Inc.—http://stereologer.com