Myths and truths about the cellular composition of the human brain: a review of influential concepts

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Abstract

Over the last 50 years, quantitative methodology has made important contributions to our understanding of the cellular composition of the human brain. Not all of the concepts that emerged from quantitative studies have turned out to be true. Here, I examine the history and current status of some of the most influential notions. This includes claims of how many cells compose the human brain, and how different cell types contribute and in what ratios. Additional concepts entail whether we lose significant numbers of neurons with normal aging, whether chronic alcohol abuse contributes to cortical neuron loss, whether there are significant differences in the quantitative composition of cerebral cortex between male and female brains, whether superior intelligence in humans correlates with larger numbers of brain cells, and whether there are secular (generational) changes in neuron number. Do changes in cell number or changes in ratios of cell types accompany certain diseases, and should all counting methods, even the theoretically unbiased ones, be validated and calibrated? I here examine the origin and the current status of major influential concepts, and I review the evidence and arguments that have led to either confirmation or refutation of such concepts. I discuss the circumstances, assumptions and mindsets that perpetuated erroneous views, and the types of technological advances that have, in some cases, challenged longstanding ideas. I will acknowledge the roles of key proponents of influential concepts in the sometimes convoluted path towards recognition of the true cellular composition of the human brain.

1. Introduction

Our understanding of the human brain – and of any brain – is based on series of facts and findings that are reported in the scientific literature. Some of the original findings were made

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decades ago, and typically have been scrutinized in subsequent scientific studies using increasingly sophisticated methodologies. A major mechanism of scientific progress is the replication and acceptance, or refutation and refinement of original findings, with evaluation by peers prior to and after incorporation into the scientific literature. This is how scientific concepts eventually become common knowledge and lay the foundation and framework for further research. The review and acceptance of new facts by peer-review is an indispensable tool of scientific advance that validates scientific knowledge and is supposed to be self-correcting (Committee on the Conduct of Science, 1989; Ioannidis, 2012). It is common that technological advances challenge old concepts and occasionally lead to refutation or amendments. Accordingly, some concepts remain in flux, to be molded by future investigators.

The human brain poses special challenges for quantification of its cellular composition. This is due not only to its large size, the large number of cells involved, and the surprisingly large variation in cell numbers between individuals, but also to the fact that, for obvious ethical reasons, researchers have much less control and access to well-preserved human brains as compared to animal brains (Peters et al., 1998; Pannese, 2011). The latter constraint has consequences for the quality and morphology of the tissues and their optimal processing for quantitative analyses.

Although studies attempted to quantify aspects of the human brain as early as the 1800s (reviewed in: Haug, 1986; von Bartheld et al., 2016), the first serious and systematic studies, with increasingly powerful methodologies, appeared about 50 years ago. Parts of the human brain were quantified using one of four basic types of methodologies, as recently reviewed (von Bartheld et al., 2016): 1. Histology of thin sections for counting of stained cell profiles, with estimates adjusted by correction factors (Abercrombie, 1946; Clarke and Oppenheim, 1995); 2. Stereology (design-based methods) using thick sections and systematic random sampling (Pakkenberg and Gundersen, 1997; Schmitz and Hof, 2005; Geuna and Herrera-Rincon, 2015); 3. DNA extraction to calculate total DNA and extrapolation to estimate cell numbers (Heller and Elliot, 1954; Dobbing and Sands, 1973); and 4. Collection of nuclei in suspension, and calculation of cell numbers based on volume samples (Nurnberger and Gordon, 1957; Johnson and Erner, 1972), an approach that was recently optimized with the design of the isotropic fractionator (Herculano-Houzel and Lent, 2005; Azevedo et al., 2009; Herculano-Houzel et al., 2015).

In this review, I examine the origin, original evidence, replication or challenges, arguments, and current status of some of the most influential concepts and questions about the quantitation and cellular composition of the human brain. I provide the historical context, explain the arguments, evaluate or assess the quality of evidence, and categorize the concepts as either refuted, confirmed, questionable or unsure when the evidence is conflicting and further research is needed. I refer to the original publications that form the basis of my evaluation so that readers can verify statements and can confirm conclusions by consulting relevant sources.

2.1. The concept of a 10:1 glia-neuron ratio (GNR)

The concept of a 10:1 GNR for the entire human brain has dominated the field over the last 50 years (Fig. 1). It originated with Holger Hyden's assertions in the 1960s (Hyden, 1960, 1967) (Fig. 2A), probably based on his model system of the vestibular nuclei in the brainstem which indeed have a large GNR. The claims of an overabundance of glia over neurons were not backed up by experimental evidence, and this may have contributed to the fact that the claims evaded customary peer review. Despite having an unvalidated status, the concept was incorporated into all major textbooks, including the most prominent books by leading neuroscientists such as Stephen Kuffler and Eric Kandel (Fig. 2B, C) (Kuffler and Nicholls, 1976; Kandel and Schwartz, 1981, 1985; Kandel et al., 1991, 2000; reviewed in: von Bartheld et al., 2016), thereby allowing the erroneous concept of a 10:1 GNR to become "common knowledge" for five decades. Instances of conflicting evidence reported by others were rare (e.g., Haug, 1986; Andersen et al., 1992), and they were basically overlooked. Different components of the brain have different GNRs (for reasons yet to be determined), and accordingly the GNR for the entire human brain is a composite of the different GNRs for its individual parts. Since GNRs are strongly conserved in evolution, they are thought to be physiologically important (Herculano-Houzel, 2014). A common mistake was to examine just one part of the brain, and to assume that the rest of the brain is composed in the same way, which prompted false conclusions (von Bartheld et al., 2016). For these reasons, the myth of a 10:1 GNR persisted until 2009, when a novel technical advance, the isotropic fractionator, revealed and effectively communicated that the GNR was less than 1:1 in human brains (Azevedo et al., 2009) – a finding that turned out to be also supported by data obtained with histological methods, including stereology (von Bartheld et al., 2016). The report by Azevedo et al. (2009) challenged and refuted the original concept of an overabundance of glial cells, and eventually led to a reversal of the GNR from 10:1 to 0.7:1, as first advocated by Herbert Haug (1986) (Fig. 3A). The concept of a lower number of glial cells than neurons in human brains is now becoming increasingly accepted in the field (Hilgetag and Barbas, 2009; Lent et al., 2012; Yuhas and Jabr, 2012; Zorzetto, 2012; Jarrett, 2015; von Bartheld et al., 2016) (Fig. 1).

2.2. The concept of 1–50 trillion glial cells in the human brain

Glial cells are smaller than most neurons and therefore are more difficult to identify and count in histological sections than neurons. Information about glial cell numbers has always been less certain than information about neuron numbers. However, the ratio between glia and neurons could be determined with a reasonable degree of certainty in distinct regions of the brain. Neuron numbers were estimated within relatively narrow ranges for certain brain regions; however, no stereological study has yet attempted to estimate glia numbers for the entire human brain. Therefore, prior to the development of the isotropic fractionator counting method, glia numbers for the whole brain were largely based on the presumed fact of a 10:1 glia-neuron ratio (GNR), which was used for calculations. Accordingly, glia numbers were postulated for the whole brain to be at least 10 times the number of neurons (which was known to be about 85–100 billion), yielding numbers of 1 trillion or more for glial cells. Several prominent textbooks increased that number by an additional factor of 10–50, notably the Kandel text book editions from 1981 and 1985 (Kandel and Schwartz, 1981, 1985), where the neuron number was erroneously stated at 1 trillion, so that a total of up to

50 trillion glial cells was postulated to exist in the human brain. These numbers conflicted with histology-based estimates of maximally 130 billion glial cells (Blinkov and Glezer, 1968) or 50 billion glial cells (Haug, 1986), but these discrepancies were not discussed in the textbooks or reviews. It was not until 2009 that the dogma of abundant glia numbers was challenged, in the paper by Azevedo et al. (2009), when the isotropic fractionator technique revealed no more than 85 billion non-neuronal cells in the human brain, with only a fraction of these – albeit a major fraction – being glial cells. Thus, the new methodology supported the estimates of 40–50 billion glial cells, as published by Herbert Haug (1986) (Fig. 3A). His estimate has been shown to be plausible based on both histological evidence as well as evidence from the isotropic fractionator, which was recently compiled (von Bartheld et al., 2016). Accordingly, the total glia number in human brains is now thought to be about 40–50 billion glial cells, which is a small fraction of the 1–50 trillion postulated in major text books and reviews just a few years ago.

2.3. The concept of a 5:3:1 ratio for neurons, glia, endothelial cells in the human brain

In the history of cell counting, a consensus about the total number of neurons in the human brain developed earlier than for glial cells. The review of Williams and Herrup (1988) provided a plausible number of about 85 billion neurons. This number of just under 100 billion neurons was widely reported in peer-reviewed articles and text books (Soper and Rosenthal, 1988; von Bartheld et al., 2016). It was supported by studies using histological/ stereological techniques (Haug, 1986) as well as isotropic fractionator methodology (Azevedo et al., 2009; Andrade-Moraes et al., 2013), a technique that was developed in 2005 (Herculano-Houzel and Lent, 2005) (Fig. 3C). Once a plausible number of glial cells in the human brain had been established at about 40–50 billion, the remaining major cell type left to be quantified were the endothelial cells. Since the number of endothelial cells is essentially the difference between the total non-neuronal cells and the total glial cells, this number can be calculated by simple subtraction. Alternatively, it can be determined by histological analyses, when percentages of glial cells and endothelial cells are compiled separately based on histological appearance (Garcia-Cabezas et al., 2016). Such percentages are tissue-specific and range from 30% of all non-neuronal cells in neocortex (= 23.6% of *all* cells in neocortex; Nurnberger, 1958; Blinkov and Glezer, 1968; Brasileiro-Filho et al., 1989; Bjugn and Gundersen, 1993; Lyck et al., 2009; Garcia-Amado and Prensa, 2012; Bahney and von Bartheld, 2014) to 85% of all non-neuronal cells in the cerebellum (a relatively high percentage, because of the low glia number) (= 16–19% of *all* cells in the cerebellum, Andersen et al., 1992; Andersen et al., 2012; Azevedo et al., 2009; Andrade-Moraes et al., 2014). Contrary to the notion that endothelial cell numbers are negligible (Bass et al., 1971; Herculano-Houzel, 2011), it is now thought that endothelial cells in the whole human brain make up about 25% of all non-neuronal cells, with the rest (75%) of non-neuronal cells being glial cells, thus generating a ratio of about 5:3:1 for neurons, glia and endothelial cells in the human brain (Bahney and von Bartheld, 2017) (Fig. 4).

2.4. The concept of loss ("fall-out") of cortical neurons with normal aging

The concept that humans lose a substantial number of cortical neurons during normal aging ("neuronal fall-out") was based on cell counting studies in the 1950s to 1980s (Brody, 1955; Brody, 1970; Colon, 1972; Hanley, 1974; Devaney and Johnson, 1980; Henderson et al.,

1980; Curcio et al., 1982; Anderson et al., 1983). Animal studies had suggested that at least some aged animals have significantly reduced numbers of neurons in their brains when compared to younger animals (Johnson and Erner, 1972; reviewed by Hanley, 1974), and Brody's and other's cell counting studies appeared to confirm this for human neocortex. These studies indicated that between 35% and 55% of cortical neurons were lost during adulthood, corresponding to a nearly 1% loss per year, and additional studies by Devaney and Johnson (1980), Henderson et al. (1980), and Anderson et al. (1983) reported similar losses (Fig. 5). Accordingly, this fall-out of about half of all cortical neurons was deemed in the 1970s and 1980s to be a normal consequence of aging, and senility was seen as an inevitable consequence of cortical neuron loss, which was thought responsible for the expected decline in intellectual abilities (Anderton, 1997; Kausler et al., 2007; Pannese, 2011). This made for a depressing outlook on life for senior citizens. Although correlation alone cannot be regarded as evidence for causation, it may not be a coincidence that suicidal ideation in the elderly spiked in the 1970s and 1980s, since mental decline is among the disabilities most feared in old age (Meehan et al., 1991; McKeown et al., 2006; Schmutte et al., 2009; Deary, 2012).

At the time when fall-out of cortical neurons was the prevailing concept, Herbert Haug (Fig. 3A) examined cell numbers in human brains and discovered that brains from older individuals had different patterns of shrinkage during fixation than younger brains (Haug, 1980; Haug et al., 1984). His re-examination of the studies claiming cell loss with normal aging showed that the apparent neuron loss was almost entirely due to a technical artifact: age-dependent differences in shrinkage, with a change in neuronal density, but not neuronal numbers. Knowledge about shrinkage is essential when neuronal densities rather than absolute numbers of neurons are examined. When corrected for actual shrinkage, the older brains showed essentially the same numbers of neurons as the younger brains (Haug et al., 1984). Haug's work corrected a major concept that had misled researchers as well as lay people (Fig. 5).

Subsequent studies have confirmed that there is either no loss, or a much smaller loss, of cortical neurons during normal aging (Terry et al., 1987; Pakkenberg and Gundersen, 1997; Peters et al., 1998; Pannese, 2011). The work of Haug (1987), Leuba and Kraftsik (1994a) and Gomez-Isla et al. (1996, 1997) found no loss, while Terry et al. (1987), Pakkenberg and Gundersen (1997), and Pelvig et al. (2008) reported a minor decrease of less than 10% during normal aging. When one combines the data from all available stereology studies into one large meta-analysis dataset (Fig. 6B, for details of the regression analysis, see legend to panel B), it becomes obvious that there is only a small decrease (2–4%) of the number of cortical neurons with age, in both males and females. The numbers for males are 24.6 ± 4.2 billion (standard deviation) for ages 20–50 vs. 23.6 ± 3.4 billion for ages 60–86; and the numbers for females are 20.6 ± 3.6 billion for ages 20–50 vs. 19.5 ± 3.6 billion for ages 70–105 (Fig. 6B). Several additional factors may be responsible for the differences between results when compared with the earlier studies: (1) large variability between individuals, making large cohorts necessary; (2) some cohorts may have included individuals with preclinical stages of Alzheimer's disease where neuron loss precedes clinical signs, although the recent work of Andrade-Moraes et al. (2013) argues against this possibility; (3) secular (generational) changes in neuron numbers may explain some of the differences in neuron

number when younger and older brains are compared; (4) abnormal diploid neuron fall-out may contribute to a small decline in neuron numbers (Fischer et al., 2012). In the most recent studies, very old females (94–105 years old) were found to have surprisingly large (and presumably stable) numbers of cortical neurons (Fabricius et al., 2013; Walloe et al., 2014). These old individuals may be a subpopulation with increased cell numbers at birth that correlate with, or may cause, longevity. As detailed by Peters et al. (1998) and Pannese (2011), changes in neuron numbers would ideally be determined in a longitudinal study, in vivo, but that is not technically possible. Based on the more recent reports and the meta-analysis presented in Figure 6B, it now appears well-established that cortical neuron numbers in humans decline very little during normal aging, and intellectual decline with age may rather be due to numerical reductions of relatively small, specific neuronal populations, or changes in aging neurons' chemistry or morphology (Morrison and Hof, 1997; Peters et al., 1998; Uylings and de Brabander, 2002; Hof and Mobbs, 2009; Pannese, 2011).

2.5. Do female brains have significantly fewer cortical neurons than male brains?

Reports are conflicting whether female human brains have fewer cortical neurons than male brains. Haug (1987) presented data indicating that female brains were smaller but had a higher density of cortical neurons, resulting in similar numbers of neurons between the genders, while Pakkenberg and Gundersen (1997) concluded that male brains have about 15% more neurons in the cerebral cortex than female brains. It is possible that the discrepancy between conclusions is due to differences between techniques: Haug used an early version of stereology (volume estimation and sampling of densities), while Pakkenberg and Gundersen applied one of the newly developed, now conventional stereological methods: the optical disector. Haug presented data for a total of 78 brains (60 male and 18 female), while Pakkenberg and Gundersen examined a total of 94 brains (62 male and 32 female). When one combines the 1997 data with those from three additional stereology studies – Braendgaard et al., 1990 (five male brains), Pelvig et al., 2008 (13 male brains and 18 female brains), and Fabricius et al., 2013 (23 female brains) – it becomes apparent in this meta-analysis of 80 male brains and 73 female brains that the stereological studies support a gender difference of about 16% (23.7 billion cortical neurons in males vs. 19.8 billion cortical neurons in females) (Fig. 6B). This difference is maintained when it is taken into account that up to seven of these brains were used in more than one study (see legend to Fig. 6B). The work of Leuba and Kraftsik (1994a) examined the visual cortex of 17 adult brains (ages 18–96 years, 10 male, 7 female), and Gomez-Isla et al. (1996) examined the entorhinal cortex, with an age range from 60–89 years (4 male, 6 female). In these studies, no differences between genders were apparent, but the numbers of subjects were too low to be conclusive.

2.5.1. Absolute numbers of cortical neurons—Figure 6 shows that the total number of cortical neurons differs significantly between Haug's 1987 study and the subsequent stereology studies; Haug's estimates were between 10 and 20 billion, while the stereology work estimated between 15 and 30 billion. Interestingly, the third method of cell counting, the isotropic fractionator (IF), reports estimates that are comparable to Haug's numbers rather than the later stereology data. However, relatively few normal brains have been examined with the IF to date (Fig. 6C). One of the two IF studies, in 2009, examined four

normal brains, from 50–71 year-old males (Azevedo et al., 2009), while the other study examined five normal brains, from 71–84 year-old females (Andrade-Moraes et al., 2013). These studies reported 16.3 billion cortical neurons for the group of younger males, and 12.7 billion neurons for the older female group: a difference of 22%. Based on Pakkenberg and Gundersen's (1997) study and the meta-analysis shown in Figure 6B, these differences in numbers seem to reflect mostly gender, rather than age differences. The stereology study by Pakkenberg and Gundersen (1997) estimated 22 billion cortical neurons for male brains, and 19 billion for females. This is at the high end of numerical estimates for cortical neurons (von Bartheld et al., 2016). The studies of Haug (1987), Herculano-Houzel (Azevedo et al., 2009) and Lent (Andrade-Moraes et al., 2013) reported lower numbers: Haug estimated about 15 billion for males, Herculano-Houzel about 16 billion for males, and Lent about 13 billion for older females. Although these numbers are consistent with a gender difference, the number of subjects examined with the isotropic fractionator (IF) is too small to conclusively confirm the results from the histological studies, especially in light of the considerable variability between individuals. Wide ranges between individuals were shown in the data of Haug (1987), West (1993a) and Pakkenberg and Gundersen (1997), with a variability that greatly exceeded the small difference between ages. The gender difference appears to be robust, since it is apparent in the later stereology studies and presumably also with the IF approach (Fig. 6B, C). Accordingly, it is likely, but has yet to be conclusively shown, that female brains have about 15% fewer cortical neurons than male brains.

2.5.2. Brain size, body size, intelligence and gender—After 180 years of research, it is now well-established that female human brains are significantly smaller, on average about 60–150 g, than male human brains (Tiedemann, 1836; Haug, 1984, 1987; Ankney, 1992; Rushton and Ankney, 2009; Deary, 2012; Pietschnig et al., 2015), but the degree to which this difference persists when adjusted for body height or size is less clear (Ankney, 1992; Rushton and Ankney, 2009; Pakkenberg and Gundersen, 1997). The question whether brain size correlates positively with intelligence (and the direction of causality) has been a matter of considerable debate (Tiedemann, 1836; Ankney, 1992; McDaniel, 2005; Rushton and Ankney, 2009; Pietschnig et al., 2015; Saini, 2017). Based on the more recent meta-analyses and reviews that take into account publication and recruitment bias (Deary, 2012; Pietschnig et al., 2015), it has been concluded that the major difference in intelligence between genders is with variance (more males than females distribute at the extremes), while the mean is virtually identical between genders (Jensen, 1980; Ankney, 1992; Deary, 2012; Pietschnig et al., 2015). In this context, recent work on the Neanderthal brain is of interest. Neanderthal brains are thought to have been about 10% larger than the brains of anatomically modern humans (Jerison, 1973; Roth and Dicke, 2005), assuming that brain mass estimates based on endocranial volumes are accurate (see Ridgway et al., 2016, for a recent challenge), yet the intelligence of the large-bodied and big-brained Neanderthals is thought to have been similar, if not inferior to Homo sapiens (Wynn and Coolidge, 2011; Villa and Roebroeks, 2014). One interesting solution to this apparent paradox is that the Neanderthal brain may have been larger because of certain specializations, such as an enlarged visual cortex, that evolved to adapt to low light intensity at high latitudes (Pearce et al., 2013), while the smaller modern humans may have had superiority in parts of the brain devoted to social cognition (Pearce et al., 2013; Villa and Roebroeks, 2014) and social skills

(prosociality, Hare, 2017). However, it is controversial whether sociality or diet drives cognitive complexity and larger brain sizes (DeCasien et al., 2017), and how this makes species more adaptive to a changing environment and ultimately more successful (Wynn and Coolidge, 2011; Villa and Roebroeks, 2014). Likewise, it is thought that some forms of cognition (e.g., visuospatial recognition), where males have superiority, require larger brain capacity (Ankney, 1992; Rusthon and Ankney, 2009). This may explain why males have increased brain sizes over females. Brain size correlates positively with cortical neuron numbers according to Haug (1987, r = 0.48) and Pakkenberg and Gundersen (1997, r = 0.56). Since brain size correlates with cortical neuron number, and brain size correlates with body size (Haug, 1984; Rushton and Ankney, 2009), one would expect that cortical neuron number correlates with body size. However, the data on this correlation is conflicting (compare Pakkenberg and Gundersen, 1997, with Rushton and Ankney, 2009). Haug (1987) found that the smaller female brains had a higher density of cortical neurons, resulting in similar numbers of cortical neurons as their male counterparts, while Pakkenberg and Gundersen (1997) found that female cortical neuron numbers were about 15% lower than those in males, so this issue is not yet resolved. It is also possible that instead of neuron number, the number of synapses and/or circuits is the crucial parameter for intelligence. As recently reviewed by Dicke and Roth (2016), studies in this respect are controversial, and reliable studies on numbers of synapses are essentially lacking. Accordingly, proper assessment of this interesting aspect has to await further work.

2.6. The concept that excessive alcohol consumption kills cortical neurons

It was widely believed in the 1970s and 1980s that alcoholism caused cortical neuron loss and behavioral dysfunction due to that neuron loss (Courville, 1955; Harper, 1983; Harper and Kril, 1985; Harper et al., 1985, 1987; Kril and Harper, 1989; Harper and Kril, 1990). Such beliefs were reinforced by studies showing significant loss of neurons in brains of animals exposed to alcohol (e.g., Walker et al., 1980). However, in humans, neuropathological studies reported that white matter of chronic alcoholics seemed more reduced than gray matter (Harper et al., 1985) and suggested regional differences in the loss of neurons (Kril and Harper, 1989; Kril and Halliday, 1999). Using the newly-developed stereological counting methods, the group of Bente Pakkenberg (Fig. 3B) reported that the total number of cortical neurons was not significantly reduced with excessive alcohol consumption (Jensen and Pakkenberg, 1993). This finding received attention, because it challenged the previously established concept of significant cortical neuron loss due to chronic alcohol abuse. However, the 1993 study only examined global (cortex-wide) loss, and was not designed to detect relatively small losses that may be restricted to distinct cortical regions, layers or cell types. Subsequent stereological studies that focused on such regional changes revealed that some cortical and subcortical regions were vulnerable to alcohol-induced neuronal losses, while other regions, such as motor cortex and hippocampus – in contrast to results from previous animal studies (Walker et al., 1980) – were not affected (Harding et al., 1997). Accordingly, a more refined concept of alcohol-induced neuronal loss now prevails (Kril and Halliday, 1999): While white matter atrophy dominates, gray matter is differentially affected, with neuron losses primarily in frontal cortex. Additional losses of gray matter are seen in certain conditions as a result of alcohol abuse: Wernicke's encephalopathy and Korsakoff syndrome are associated with neuron losses in the

hypothalamus, thalamus, and cerebellum (Harper and Matsumoto, 2005). This refined concept of regional, restricted losses provides more hope to former and recovering alcoholics, because axonal (white matter) and myelination deficits may be potentially reversible, while dead neurons in the central nervous system generally cannot be replaced.

2.7. Are there secular (generational) changes in cell numbers in human brains?

Herbert Haug pointed out that some of the reported changes in cell numbers in normal aging brains may be due to generational changes in bodies and brains of the population, as body height increased from the 20th to the 21st century (Haug, 1984; Haug, 1987; reviewed by Peters et al., 1998), possibly due to improved nutrition. Several studies claim that body height is positively related to brain weight (reviewed in Rushton and Ankney, 2009), and Haug assumed that therefore, body height was also positively related to cortical neuron number (Haug, 1987). However, surprisingly, Pakkenberg and Gundersen (1997) concluded that cortical neuron number was not significantly related to body height, at least not when this was calculated separately for each gender. This has interesting implications, as Pakkenberg and Gundersen (1997) observe: "body size … has no relationship to neocortical neuron number for individuals of a particular sex: small men may have large neocortical neuron numbers, and large men may have small neocortical neuron numbers," presumably because neuron number is determined very early in life, while body height manifests 15 to 20 years later, and is in part determined by environmental influences. It should be noted that these findings disagree with the concept of the encephalization quotient (Jerison, 1973), as recently discussed (Herculano-Houzel, 2017). Nevertheless, the apparent lack of correlation between body height and cortical neuron number in humans undermines Haug's idea about secular changes as a relevant factor to explain some studies' neuron loss with age. Even though Haug did not find any significant loss of neurons in older individuals in his own data, he cited secular acceleration as a factor that may make it look as if cortical neurons are lost, as had been reported by others (Brody, 1955; Brody, 1970; Colon, 1972; Hanley, 1974; Devaney and Johnson, 1980; Henderson et al., 1980; Anderson et al., 1983), because younger individuals with a higher base level (larger number of neurons) are compared to individuals with a lower base level (lower number of neurons) that is not due to loss of neurons (Peters et al., 1998). The meta-analysis presented in Figure 6B indicates rather small numerical differences (decreases of about 3%) in cortical neurons between cohorts with increasing age. Although body height and brain weight are undergoing secular changes in most populations, with mean body height increasing by 1 mm per year and brain weight by 50–60 g per century, changes which appear to have been occurring only in the last 200 years (Haug, 1984; Haug, 1987; Miller and Corsellis, 1977), one must take into account that it is not clear whether cortical neuron number correlates with body-size and height, given the negative results by Pakkenberg and Gundersen (1997). Accordingly, Haug's concern about the effect of secular acceleration for "pseudo" neuron loss in normal aging is now mitigated for two reasons: (1) there is minimal loss of cortical neurons with normal aging, and (2) there may not be a relation between body height and cortical neuron number. Relative stability of neuron numbers with age has also been documented for subcortical human neuronal populations, such as brainstem catecholaminergic neurons (Mouton et al., 1994; Kubis et al., 2000) and the cochlear nucleus (Sharma et al., 2014).

2.8. Do glia make you smart?

There has been a longstanding notion, based on comparisons between vertebrate species, that increased numbers of glial cells in the brain correlate with, and possibly contribute to, superior intelligence. As discussed in the section 2.5.2, the relationships between brain size, neuron number, and intelligence are still controversial. The GNR in cerebral cortex was observed to increase with increasing brain sizes between species, suggesting a correlation and possible causation between high GNRs and superior intelligence (Friede, 1954; Jerison, 1973; Araque et al., 2001; Fields, 2009; Koob, 2009; Verkhratsky and Butt, 2013). The idea gained further traction when Albert Einstein's brain was found to contain normal neuron numbers, but appeared to have slightly increased glia numbers in some cortical regions (Diamond et al., 1985; Witelson et al., 1995), although the methodology of estimating cell densities based on profile counts is problematic according to current knowledge. Furthermore, animal studies showed that injecting human glia into mouse brains made those mice smarter in learning tests (Han et al., 2013; Fields, 2013). However, the notion of an increasing GNR with increasing brain mass (supported by Stolzenburg et al., 1989; Nedergaard et al., 2003; Marino, 2006; Sherwood et al., 2006; Bahney and von Bartheld, 2017) has recently been questioned (Herculano-Houzel et al., 2007; Herculano-Houzel, 2011). An alternative interpretation of the GNR's correlation with intelligence is that the correlation is with increasing brain size and not necessarily intelligence. This was supported by studies on whale brains, which showed even higher GNRs than human brains (Hawkins and Olszewski, 1957; Tower and Young, 1973; Haug, 1987; Eriksen and Pakkenberg, 2007). The concept was further clarified by comprehensive scaling studies using the isotropic fractionator: such studies indicated that the GNR depends primarily on neuron size (which is related to brain size and also neuron density), and is not determined by intelligence per se (Herculano-Houzel, 2011, 2015). Furthermore, the spinal cord's GNR increases similarly or even more with increasing brain size than cortical GNRs (Bahney and von Bartheld, 2017), showing that not only cortical GNRs increase with brain size, but also subcortical and even spinal cord GNRs. Thus, the reason for GNR increases with increasing brain size does not appear to be directly linked to intelligence.

2.9. Should all cell counting methods be calibrated, even when they are theoretically unbiased? How can biases be identified and avoided?

It became apparent in the 1980s that cell counting methods can generate widely diverging results (Haug, 1986; Schmalbruch, 1987, Table 1). This gave rise to the idea that these methods should be validated by calibration (Schmalbruch, 1987). To address this problem, comparison with a serial section reconstruction – the so-called "gold standard" – was considered to be the most useful approach. In this context, calibration means that estimates obtained by a histological counting method are directly compared with the true number of particles in that same tissue obtained by exhaustive counting in a 3D serial section reconstruction (Coggeshall et al., 1990; Pover and Coggeshall, 1991). Such a comparison would show whether a method is biased, and if so, by how much. Accordingly, any bias of a counting method can be assessed and the method thereby validated – although it has to be kept in mind that numerous parameters can contribute to bias (as discussed below). For the isotropic fractionator (IF), validation is not as straightforward, because the same tissue cannot be used for direct comparison as for the histological counting methods. Therefore,

the IF can only be compared with other, preferably already validated, counting methods. One needs to avoid validation that is "circular", such that method A is validated with method B, and method B is validated by comparison with method A. This is not the case in the examples discussed below, because stereology was validated against an exhaustive count (3D serial reconstruction), the "gold standard", and then the IF was compared with the (already validated) stereology method, thus preventing circular reasoning.

Coggeshall and his colleagues pioneered the validation approach by first calibrating various 2D methods (Coggeshall et al., 1990), followed by calibration of the physical disector (Pover and Coggeshall, 1991). Coggeshall realized that such a calibration need not to be performed on an entire peripheral ganglion or nucleus in the brain, which would be prohibitively time-consuming to process and analyze in serial sections, but that a small sample of tissue sections could be serially reconstructed as the gold standard to validate counting methods (Coggeshall et al., 1990; Pover and Coggeshall, 1991; Coggeshall, 1992; Farel, 2002; Delaloye et al., 2009). Such analyses proved effective in revealing practical features important in minimizing biases in the counting methods (Pover and Coggeshall, 1991; Popken and Farel, 1996; Hatton and von Bartheld, 1999; von Bartheld, 2001, 2002). When the physical disector was calibrated, it was discovered that estimates generated from disectors with adjacent reference and look-up sections require an optimal distance between the consecutive disector pairs (Pover and Coggeshall, 1991; Delaloye et al., 2009). Pover and Coggeshall emphasized that "we would not have known this was a problem if we did not verify our counts." Likewise, when Farel and colleagues calibrated the physical disector, they found that the recommended sampling of 100–200 particles generated an unacceptable variability, and that sampling of over 300 particles was necessary (Farel, 2002). When Hatton and von Bartheld (1999) calibrated the optical disector, they found that the use of recommended guard zones introduced a deficit of over 20% in numerical estimates, apparently due to compression of the margins of soft tissue sections – the unsampled guard zones (Baryshnikova et al., 2006).

Historically, stereologists have had a mixed reaction to efforts of calibrating the new counting methods. Some, such as Mark West (West, 1999), Rob Williams (Williams et al., 2003) and Jens Nyengaard embraced this approach (Basgen et al., 2006: "We consider the Exhaustive Count method to be the gold standard for cell counting, since there is no sampling … and every nucleus is counted directly"), while others, such as Cruz-Orive, Gundersen, and Howard claimed that the new stereological methods did not need calibration, because they were inherently (theoretically) unbiased (Cruz-Orive, 1994; Mayhew and Gundersen, 1996; Howard and Reed, 1998). This notion prompted extensive discussions and concerns about a "double standard" – why would only 2D methods need validation, but not 3D methods (Guillery and Herrup, 1997; Saper, 1999; Benes and Lange, 2001a; von Bartheld, 2001; von Bartheld, 2012)? Table 2 compiles studies that calibrated 2D and 3D methods as well as the IF. As detailed below, these efforts have led to the identification of several types and sources of potential bias, some of them generally applicable (relevant for all counting methods), while others are specific to certain methodology.

2.9.1 Issues with counting methods – some revealed by calibration analyses

<u>General issues:</u> Particles to be counted require unambiguous identification and clear inclusion criteria (Ward et al., 2008; Kaplan et al., 2010), specifically for the distinction between glia and small neurons (Flood, 1994; von Bartheld et al., 2016). The region of interest needs a clear delineation and/or definition (Thune and Pakkenberg, 2000; Dorph-Petersen and Lewis, 2011, 2017). This is straightforward for nuclei with well-defined borders, but can be challenging for nuclei with diffuse or geometrically bizzare borders, e.g., the nucleus subceruleus (von Bartheld and Bothwell, 1992), or the mesencephalic nucleus of the trigeminal nerve (von Bartheld and Bothwell, 1993). Operator and inter-observer variability and bias, including errors in the implementation of counting procedures and generation of estimates, need to be minimized (Guillery, 2002; Mouton, 2002; Kaplan et al., 2010; Dorph-Petersen and Lewis, 2011).

<u>Stereology-specific (mostly thick-section) issues:</u> Regarding the originally recommended sampling scheme in which only 150–200 particles are counted (Braendgaard et al., 1990; Coggeshall and Lekan, 1996; Howard and Reed, 1998; West, 1999), it is important to note that several investigators believe that larger samples are needed, especially when the tissues are heterogeneous (Kordower, 2000; Farel, 2002; Schmitz and Hof, 2000, 2005). Measurement of section thickness is not a trivial undertaking (Guillery, 2002; Guillery and August, 2002). Dealing with differential tissue section shrinkage, and the appropriate placement of guard zones (to prevent sampling in compromised zones of tissue sections) is a highly complex issue, because tissue sections can be initially differentially deformed with subsequent loss of particles when treated in harsh conditions (Hatton and von Bartheld, 1999; Gardella et al., 2003; Baryshnikova et al., 2006; Carlo and Stevens, 2011; von Bartheld, 2012). The problem that particle distribution may be distorted in the z-axis is a potential source of bias that unfortunately is ignored in many reviews (e.g., Mouton, 2002; Dorph-Petersen and Lewis, 2011), possibly because not all types of tissues are affected. While some investigators outside the von Bartheld lab have reported such distortions (e.g., Andersen and Gundersen, 1999; Baryshnikova et al., 2006; Carlo and Stevens, 2011), others did not (Pelvig et al., 2003; Hosseini-Sharifabad and Nyengaard, 2007). The behavior of tissues in the hands of different investigators is remarkably variable, and it is not yet known why some tissue sections do not lose caps, while others show a marked loss – which was the reason to implement the now controversial guard zones in the first place (Andersen and Gundersen, 1999). For a detailed examination why distortion of tissue sections can be difficult to prove, see Baryshnikova et al. (2006).

Some proponents of nonstereological methods have stated that design-based stereology requires tissues to be homogenous, with particles distributed in a completely random fashion (Benes and Lange, 2001a; Herculano-Houzel and Lent, 2005; Herculano-Houzel et al., 2015). This is a misconception (Baddeley, 2001). It is true that heterogeneity in tissues will increase variance and will enlarge the coefficient of error (Dorph-Petersen and Lewis, 2011), making a larger number of samples necessary (Benes and Lange, 2001b). The use of relatively few samples and actual neurons counted in 3D methods has prompted questions whether stereological analysis of tissues with heterogeneous distribution of particles can be accomplished with the generally recommended sampling schemes (Benes and Lange,

2001a,b; Guillery, 2002; Guillery and August, 2002; Herculano-Houzel and Lent, 2005). For example, there have been concerns that counting only 300–400 particles in a structure as heterogeneous as cerebral cortex may not be sufficient to generate estimates of 26 billion neurons (Braendgaard et al., 1990; Benes and Lange, 2001b). The recently developed Proportionator tool (Gardi et al., 2008a,b) was designed to enhance sampling efficiency and to thereby facilitate stereological analysis of tissues with cells that are heterogeneously distributed.

IF-specific issues: The concerns with the IF counting method are that nuclear membranes may be ruptured, and destroyed nuclei may be discarded as debris, or that the neuron-specific antibody may not recognize all neuronal nuclei, both of which could lead to an undercount of nuclei (Yuhas and Yabr, 2012; Carlo and Stevens, 2013; Charvet et al., 2015). Autolytic or necrotic cells, especially in human tissues, may not incorporate fluorescent dyes or may not be recognized by antibodies, thus requiring stringent quality control, although the fixed cell nuclei appear to be remarkably robust in this respect (von Bartheld et al., 2016). Empirical studies show that superior tissue preservation is more important for morphological than for biochemical analysis, making the IF the more versatile method when tissue quality varies (Bahney and von Bartheld, 2014).

Some profile counting work has resulted in large variations between studies (Table 1), although the most egregious examples (outliers) were presumably due to no or inadequate use of correction factors (Clarke, 1992; von Bartheld, 2001). It was hoped that stereology would reduce that variability, but the results are mixed: A survey of reviews shows that numerical estimates can be within reasonably tight ranges (for example in rat dorsal root ganglia compiled by Tandrup, 2004, or in rat hippocampus compiled by Schmitz and Hof, 2005), while others are more divergent (Herculano-Houzel et al., 2015, see examples in Table 1). Therefore, it has been argued that stereology is not necessarily preferable to properly conducted profile counting (von Bartheld, 2001). How does the width of ranges yielded by stereological estimates compare with the new method, the isotropic fractionator (IF)? The IF was recently validated (Bahney and von Bartheld, 2014; Miller et al., 2014; Herculano-Houzel et al., 2015; Ngwenya et al., 2017), and so far the results are encouraging. However, it has to be kept in mind that relatively few groups have applied this new method, and variability in results can be expected as more investigators use it. To my knowledge, only two substantive challenges have emerged: Charvet et al. (2015) pointed out that IF-derived neuronal numbers for cerebral cortex, in particular the visual cortex of rhesus monkeys, was lower (by 50%) than stereology-derived numbers. However, some of the stereology-derived numbers appear to be unusually high, and the authors fail to mention corroborating lower numbers from the older literature. The second challenge (Bahney and von Bartheld, 2017) is that a series of estimates for neuron numbers in the primate spinal cord appear to have been unusually low (Burish et al., 2010), for reasons that are not entirely clear.

It may be considered fortunate that the histological and the IF methods have different types of limitations. A major problem for stereology is that small neurons are difficult to distinguish from glial cells (recently reviewed in: von Bartheld et al., 2016). Several groups have attempted to solve this problem by using neuron-specific antibodies such as NeuN that

recognize a neuronal antigen present within the nucleus of most neuronal populations (Lyck et al., 2009; Giannaris and Rosene, 2012; Zhu et al., 2015; Ngwenya et al., 2017). However, there are conflicting reports about the success of identifying neurons in tissue sections by using NeuN immunocytochemistry. When Lyck et al. (2009) compared the number of immunolabeled cells in human tissue sections with the identification of cells by morphological criteria, only 18–57% of otherwise neuronal-appearing cells were labeled by the NeuN antibody. On the other hand, Giannaris and Rosene (2012) and Zhu et al. (2015) reported that all neurons, and only neurons, were NeuN-labeled in their mammalian forebrain studies, and likewise Iwaniuk's group found the same in their chicken brain sections (Ngwenya et al., 2017). Apparently, tissue processing, cell types, and neuronal physiology may all play a role in how much NeuN is actually present and can be identified by immunocytochemistry in tissue sections (Mullen et al., 1992; Tsai et al., 2009; Carlo and Stevens, 2013), while the antibody labeling appears to work in a more robust fashion for cell nuclei in suspension (when using the IF approach).

Taken together, this survey shows that indeed, all counting methods benefit from calibration and validation. More work is needed to identify and to minimize sources of bias. It is essential that authors consider all types of potential biases, acknowledge differences in results between studies, try to resolve discrepancies by calibration analyses, discuss their new data in the context of relevant previous studies, and report all important parameters that inform how the sampling and counting was conducted and estimates were obtained (Schmitz and Hof, 2005; Dorph-Petersen and Lewis, 2011).

2.10. How does the cellular composition of the brain change in neurological and psychiatric disease conditions?

Besides the classical neurodegenerative diseases with clearly defined neuron losses, such as in Huntington's and Parkinson's disease (Vonsattel and DiFliglia, 1998; Kordower et al., 2013), several additional neurological and psychiatric diseases have been implicated with abnormal glia numbers, neuron numbers, or GNRs (Rowland and Mettler, 1949; Benes, 1993, 1997; Ongür et al., 1998; Harrison, 1999; Coyle and Schwarcz, 2000; Vawter et al., 2000; Todtenkopf et al., 2005; Bernstein et al., 2015; Elsayed and Magistretti, 2015). While the degree and localization of abnormalities differ between studies, some changes in glial cell number, densities or GNRs have been confirmed in discrete brain regions by recent studies employing stereological or IF methods for conditions including autism spectrum disorders, mood disorder, depression, schizophrenia, and Alzheimer's disease (Rajkowska, 2000; Cotter et al., 2001; Hof et al., 2003; Vostrikov et al., 2007; Morgan et al., 2010; Karlsen and Pakkenberg, 2011; Andrade-Moraes et al., 2013; Verkhratsky et al., 2014; Dorph-Petersen and Lewis, 2017).

The lack of reliability, validity and therefore trust in quantitative data has been a major impediment to progress in defining the potential roles of numerical glia or neuron abnormalities in neurological and psychiatric diseases (Williams and Rakic, 1988; Stark et al., 2005). There have been multiple examples where initial reports of numbers or ratios of glial cells and neurons could not be replicated or had to be substantially revised, even within the same group of investigators or when using the same type of counting technique (Guillery

and Herrup, 1997; von Bartheld, 2001; Herculano-Houzel et al., 2015; Dorph-Petersen and Lewis, 2017; Kaur et al., 2018). Meta-analyses have not been able to resolve all controversies about glia numbers and ratios in human neurological and psychiatric diseases (Harrison, 1999; Rajkowska, 2000, 2002; Hof et al., 2003; Lyness et al., 2003; Palmen et al., 2004; Todtenkopf et al., 2005; Courchesne et al., 2007; Amaral et al., 2008). It is beyond the scope of this contribution to review all reports of numerical glia or neuron abnormalities in neurological or psychiatric diseases. I will focus instead on the following two examples that are particularly instructive: the question of neuron loss in the thalamus of people with schizophrenia, and the question of cortical neuron loss in Alzheimer's disease.

Schizophrenia researchers have examined multiple brain regions for abnormalities of neuron and glia numbers as well as ratios between cell types such as the GNR, including various cortical regions as well as subcortical structures (Benes, 1993; Harrison, 1999; Todtenkopf et al., 2005; Dorph-Petersen et al., 2007; Bernstein et al., 2015). The dorsomedial thalamus was implicated in schizophrenia when stereological studies reported a 30–40% decrease in neuron numbers (Pakkenberg et al., 1990, 1992, 1993; Popken et al., 2000). However, subsequent studies were unable to replicate these findings, including a study by the same lead author, Bente Pakkenberg (Nielsen et al., 2008; reviewed in Dorph-Petersen and Lewis, 2017). On the other hand, the pulvinar seems to have abnormal neuron numbers in schizophrenia that are supported by the majority of studies (Dorph-Petersen and Lewis, 2017). The review by Dorph-Petersen and Lewis comprehensively examined the potential reasons for the discrepant results for the dorsomedial thalamus. Since the reviewed studies employed stereology, there should not have been – theoretically – any issues due to different technical approaches (although this still leaves observer errors and biological variation). The authors discuss the heterogeneity of the schizophrenia cases, with the initial cohorts consisting of hospitalized patients with somewhat older ages, while the subsequent studies obtained brains from younger subjects in (non-hospital) community settings. While there was no single conclusive reason for the discrepancies, this example does show how difficult it is, with the small cohorts available and possibly a large biological variability, to find convincing evidence for relatively subtle abnormalities in neuron or glial cell numbers.

In the 1980s, it seemed fairly well-established that cortical neurons, especially the larger neuron types, were significantly reduced in Alzheimer's disease, based on the work of Terry and others (Terry et al., 1981; Terry and Davis, 1983; Mountjoy et al., 1983; Price, 1986; Braak and Braak, 1986; Terry and Hansen, 1988; West et al., 1993b; Mann, 1996). It was assumed that this neuron death contributed to many clinical manifestations of the disease (Price, 1986). Therefore, it came as a surprise, if not a shock, when the first major stereological study, by Pakkenberg's group (Regeur et al., 1994), reported that there was no significant global loss (the observed loss of 6% was not statistically significant) of neurons in the cerebral cortex of Alzheimer's patients when compared with a similarly-aged control group. This study examined the cerebral cortex of 11 women with severe Alzheimer's, compared with 10 cognitively normal women of a similar age (mean of 83–84 years). This paper was published in *Neurobiology of Aging*, and because it prompted reversal of the thinking about the pathology of Alzheimer's disease, the editors invited eighteen leading Alzheimer's researchers to comment and express their thoughts about the validity and the implications of the paper in the same issue of the journal (e.g., Braak and Braak, 1994;

Flood, 1994; Hyman and Gomez-Isla, 1994; Mufson and Benzig, 1994), and other experts expressed their opinions in additional publications (e.g., Everall et al., 1997; Peters et al., 1998). While some voiced concerns about the technique, by and large the consensus was to accept the findings, because the data were generated by a so-called unbiased method (although it had not yet been calibrated at the time – see section 2.9). The main new conclusions were that (1) neuronal cortical death was restricted to certain cortical regions, so neuron losses were not apparent in a study that examined neuronal losses globally (cortex-wide); (2) neurons may shrink rather than die, so they would appear in a different size category (or even be counted as glial cells in the older studies), and (3) reductions in neurotransmitters and synapse numbers, rather than degeneration and death of cell bodies, may underlie the clinical deterioration (Peters et al., 1998; Uylings and de Brabander, 2002; Pannese, 2011). Remarkably, during the next 20 years, the findings of Regeur et al. (1994) were neither challenged nor confirmed. Some studies examined specific regions of cortex or specific neuronal populations, and found some losses (Leuba and Kraftsik, 1994b; Gomez-Isla et al., 1996, 1997). Such results were reconciled, because some investigators still believed in the only recently debunked "fallout of cortical neurons with normal aging" (see Section 2.4), and assumed that losses in older patients with Alzheimer's disease would be relatively small (because many neurons would have already been lost due to normal aging, Mann, 1996). Others believed that the specific losses were too small to be apparent as a significant global neuron loss (Duyckaerts et al., 2009).

However, in 2013, Roberto Lent's group published a study (Andrade-Moraes et al., 2013) where they used the new counting method, the IF, to examine the same question that Regeur et al. had addressed 20 years earlier: are there significant changes "globally" in neuron and non-neuronal numbers in the entire cerebral cortex of Alzheimer's patients? In this study, the brains of four subjects with asymptomatic disease and five subjects with dementia were compared with five normal controls. With the IF method, Andrade-Moraes et al. showed that there *was* a statistically significant loss (of 29%) of neurons globally in the entire cortex of demented patients, and also in specific lobes of cortex, amounting to a loss of 41% for frontal lobe, and 24% loss for the other lobes, consistent with the studies and conclusions prior to 1994 (Braak and Braak, 1986; Mann, 1996). Surprisingly, Andrade-Moraes et al., did not comment on the earlier study by Regeur et al. and did not mention the extensive debate and series of commentaries it had prompted in 1994. In fact, the Regeur study was not even cited. Possibly, Andrade-Moraes et al. (2013) were reluctant to point out the apparent discrepancy between methods. In 2013, the IF had not yet been validated and had never been side-by-side directly compared with stereology. Based on the validation studies that appeared just a short while later (Bahney and von Bartheld, 2014; Miller et al., 2014), one would have expected that IF and stereology would produce similar results. Since two convincing and supposedly methodologically equivalent studies yielded discrepant results on this important question, it now appears unclear which group and conclusion is correct. Without additional studies, it is impossible to decide whether the IF study, that corroborates the older studies and results prior to 1994, is more believable, or the 1994 stereology study. The 1994 study was conducted and published prior to the work that revealed a number of hidden biases (see above), and maybe would have faced more scrutiny if it had been known that the theoretically unbiased methods can have significant biases when applied to real

world tissue sections and constraints (von Bartheld, 2012). Obviously, we do not yet know the answer to this question, but what appeared to be generally accepted for 20 years about the persistence of cortical neurons in Alzheimer's disease now seems less certain and has to await further scrutiny and resolution.

3. Conclusions

In summary, this survey of ten influential concepts in quantitative methodology, designed to elucidate the cellular composition of the human brain, teaches several lessons. We need to be vigilant about assumptions that are taken for granted, as shown by the evidence that "text-book knowledge" may not be correct, or the hubris that calibration and validation of counting methods may not be needed. Methodologies will continue to be developed and refined, and it is of utmost importance to relate new findings to previous relevant reports, to discuss differences in results, and attempt to identify underlying mechanisms of discrepancies. It is important to keep an open mind and to be inquisitive and creative, in order to separate truths from myths and to approximate the cellular composition of brains, including that of our own species.

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Abbreviations

CNS	central nervous system
GNR	glia-neuron ratio
IF	isotropic fractionator

Highlights

- The myth of a 10:1 glia-neuron ratio in human brains has been debunked

- The myth of one trillion glial cells in human brains has been debunked

- The number of cortical neurons does not decline significantly in normal aging

- All counting methods benefit from calibration and validation

- Proof is needed for altered cell numbers in neurological and psychiatric disorders
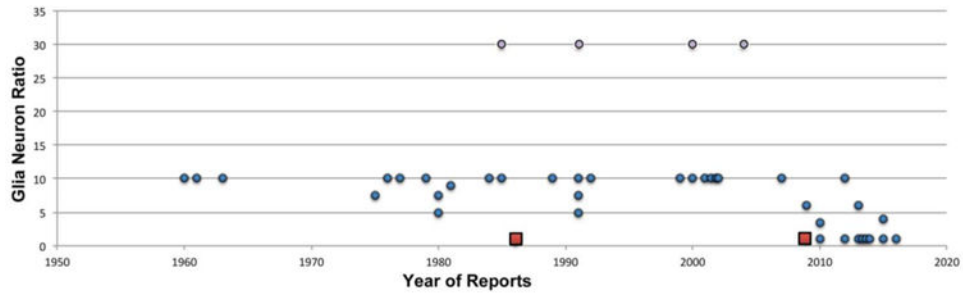
Fig. 1.
Reports of the glia neuron ratio (GNR) in human brains from the 1960s to the current time. Note the obvious outliers by Kandel's and other's text books with a GNR of 30 (10–50), indicated by purple dots, and the pioneering reports of a GNR of 0.7–1 by Haug (1986), and by Azevedo et al. (2009) that used the isotropic fractionator, indicated by red squares. Data are compiled from the Table 5 published by von Bartheld et al. (2016).
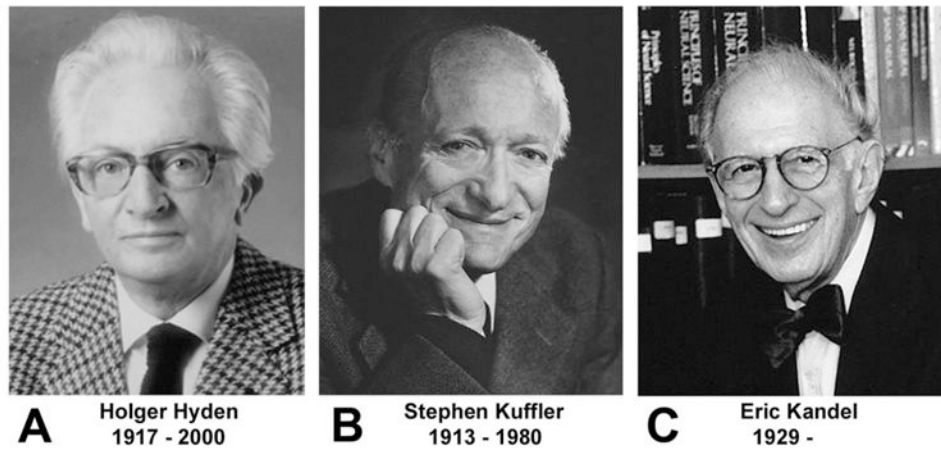
A Holger Hyden 1917 - 2000 **B** Stephen Kuffler 1913 - 1980 **C** Eric Kandel 1929 -

Fig. 2.
A-C. Photographs of individuals who had a major influence on concepts regarding the cellular composition of the human brain. This composite shows the three main proponents of the 10:1 glia-neuron ratio and the notion of one trillion glia cells in the human brain. **A.** Holger Hydén was a professor at the University of Goteborg, Sweden, and the first to claim a 10:1 glia-neuron ratio in the 1960s (Hyden, 1960, 1967). Photo: Courtesy of Anders Hamberger, photography: Lennart Nilsson. Reproduced with permission. **B.** Stephen Kuffler, professor at Harvard and the "father of modern neuroscience," was the first to promote the "at least 10:1 ratio" in his influential textbook in 1976 (Kuffler and Nicholls, 1976). Photo by Bachrach, Boston. Reproduced with permission. **C.** Eric Kandel, professor at Columbia University and Nobel laureate (2000), contributed to the perpetuation of the notion of one trillion glial cells by stating that glia outnumber neurons 10–50fold in his many editions of "Principles of Neural Science" – the "bible of neuroscience" visible in the bookshelf (Kandel and Schwarz, 1981 Kandel and Schwarz, 1985). Photo credit: Columbia University. Reproduced with permission.
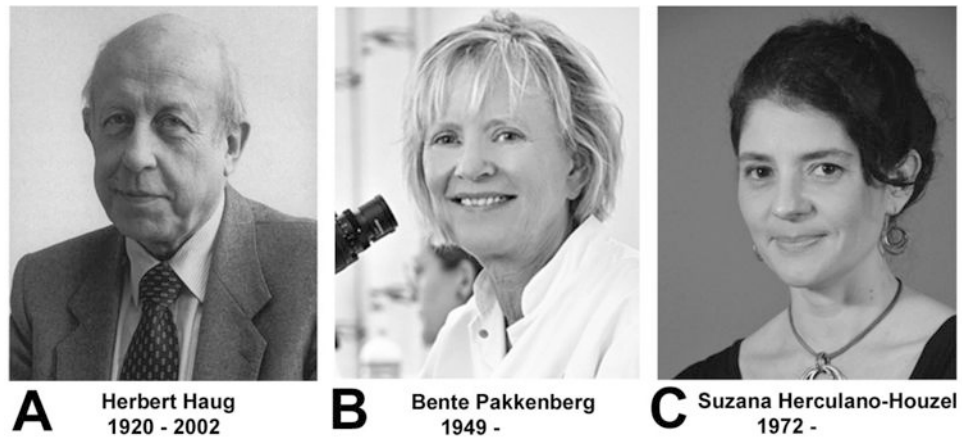
A Herbert Haug
1920 - 2002

B Bente Pakkenberg
1949 -

C Suzana Herculano-Houzel
1972 -

Fig. 3.
A-C. Photographs of individuals who had a major influence on concepts regarding the cellular composition of the human brain. This composite shows the three main investigators who's work debunked several myths, including the claim of a 10:1 glia-neuron ratio and the trillion glia myth. **A.** Herbert Haug, professor at the University of Lübeck, Germany, correctly estimated in 1986 the glia-neuron ratio to be less than 1, based on his own cell counts, and he refuted the prevailing myth of neuronal fallout with normal aging. Photograph originally published by Wolfgang Kühnel (Annals of Anatomy, 185:293, 2003), and reproduced with permission by Elsevier. **B.** Bente Pakkenberg, professor at Bispebjerg University Hospital, Denmark, a pioneer in the use of stereological methods, determined cell numbers in numerous human brain structures, including cerebral cortex, cerebellum, and the effect of aging, diseases, and alcohol use on neuron numbers in human brains. Photograph by Claus Peuckert, *claus peuckert photography*, reproduced with permission. **C.** Suzana Herculano-Houzel, professor at Vanderbilt University, developed a highly efficient alternative counting method, the isotropic fractionator. Historically, this method was the first that revealed and effectively communicated the true number of non-neuronal cells and the true ratio of non-neuronal to neuronal cells in human brains. Photo credit: Luiza Herculano-Houzel. Reproduced with permission.
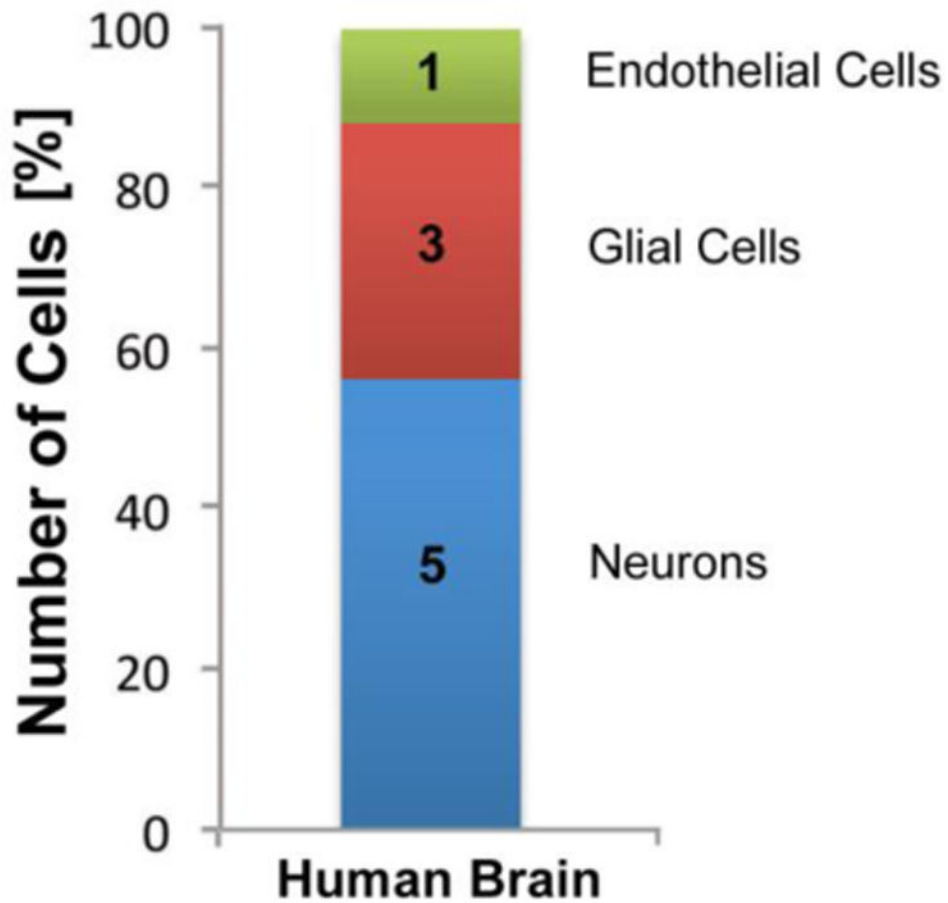
Fig. 4.
Cellular composition of the human brain: the concept of a 5:3:1 numerical ratio of neurons (blue), glial cells (red), and endothelial cells (green). Data and concept as originally designed in Bahney and von Bartheld (2017), and based on current estimates of the numbers of neurons, glia and endothelial cells (von Bartheld et al., 2016).
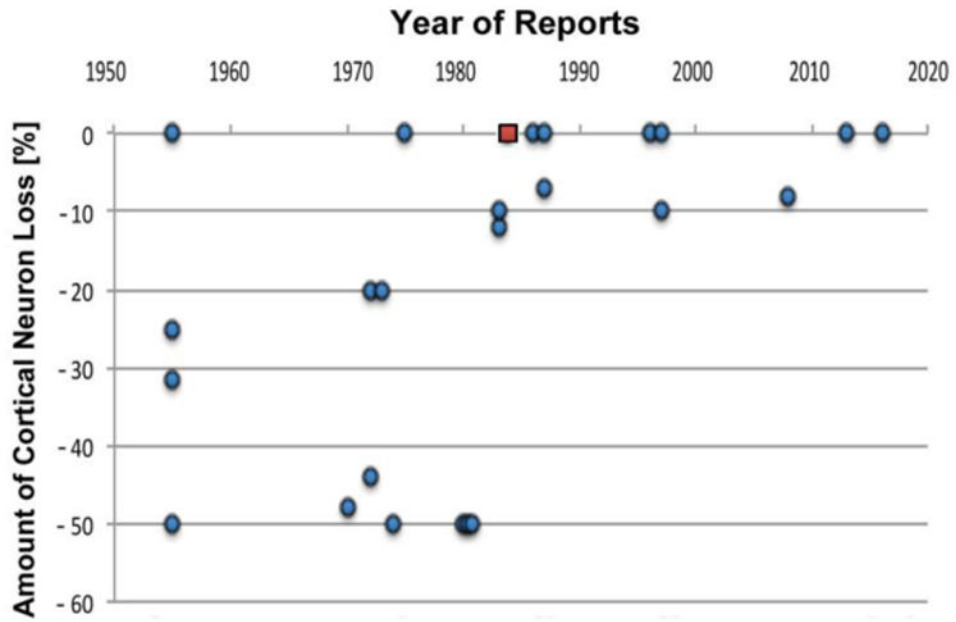
Fig. 5.
Reports of neuron death in the human cerebral cortex during normal aging. Note that in the 1950s through 1980s reports prevailed that claimed substantial neuron death ("neuronal fall-out") during normal aging, until the report of Haug et al. (1984) (indicated with a red square) convincingly exposed this concept to be a technical artifact. It is now well established that there is no significant global cortical neuron loss with normal aging (see also Fig. 6).

Fig. 6.

A-C. Graphs of the number of cortical neurons in male and female brains with normal aging, based on one study using an early form of stereology (A), four studies using convent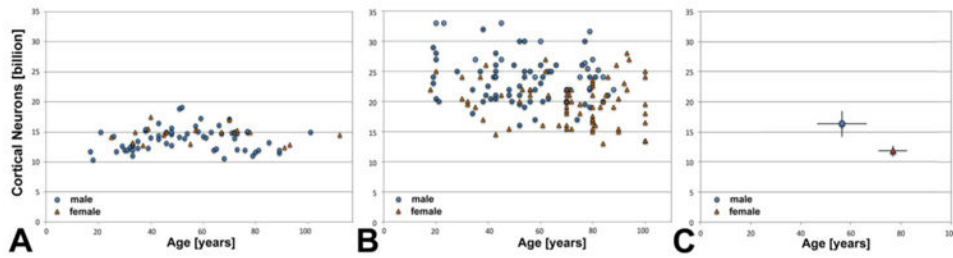ional stereology (B), and two studies using the isotropic fractionator (C). Comparison of the results of a total of seven studies: by Haug (1987) (A), (Braendgaard et al., 1990; Pakkenberg and Gundersen, 1997; Pelvig et al., 2008; Fabricius et al., 2013) (B), and the isotropic fractionator (IF) (Azevedo et al., 2009; Andrade-Moraes et al., 2013) (C). The bars in panel C denote ranges with n=4 for males, and n=5 for females; exact individual data points could not be shown for these two studies, because the data were reported only in aggregate in the original publications. Note that Haug's estimates correspond well in numbers with those obtained by the IF methodology, while the subsequent stereology estimates are somewhat higher. Note also that numbers do not decline significantly with age (panel A: $y = 13.25$ billion $+ 0.0099x$ for males, and $y = 15.0$ billion $- 0.0071x$ for females), while in panel B the regression analyses show a minor decline of 2–4% ($y = 24.6$ billion $- 0.0211x$ for males, and $y = 21.7$ billion $- 0.0245x$ for females). The regression analysis is similar when up to seven possible duplicate data points are removed in panel B (because some of the same brains were used in multiple studies): $y = 25.7$ billion $- 0.0336x$ for males, and $y = 21.8$ billion $- 0.0266x$ for females, or when the very old females (ages 94–105) from the Fabricius et al. (2013) study are omitted ($y = 21.5$ billion $- 0.0209x$). Female numbers are significantly lower (by about 15–16%) than male numbers (p<0.0005), based on Gundersen and Pakkenberg's (1997) stereology work (panel B).

Table 1

Variability in numerical estimates between quantitative studies

Nervous System Structure	Species	Method	Numerical Ranges	References
Cerebral cortex	Human	HIS	$0.6\text{-}16.5 \times 10^9$	Haug, 1986
Dorsal root ganglion, Lumbar 4	Rat	HIS	$5.3\text{-}12.5 \times 10^3$	Schmalbruch, 1987
Dorsal root ganglion, Lumbar 5	Rat	HIS	$2.0\text{-}14.8 \times 10^3$	Schmalbruch, 1987
Dorsal root ganglion, Lumbar 6	Rat	HIS	$4.1\text{-}14.1 \times 10^3$	Schmalbruch, 1987
Purkinje cells	Rat	STE	$2.1\text{-}6.1 \times 10^5$	Guillery & Herrup, 1997
Cerebral cortex	Human	STE	$13.7\text{-}22.8 \times 10^9$	Peters et al., 1998
Dentate gyrus	Mouse strains	STE	$0.38\text{-}0.94 \times 10^6$	Abusaad et al., 1999
Pyramidal cell layer	Mouse strains	STE	$0.43\text{-}0.93 \times 10^6$	Abusaad et al., 1999
Dorsal root ganglion, Lumbar 5	Rat	STE	$14.1\text{-}19.2 \times 10^3$	Tandrup et al., 2004
Hippocampus	Human	STE	$22.6\text{-}40.4 \times 10^6$	Korbo et al., 2004
Granule cells (Dentate gyrus)	Rat	STE	$1.2\text{-}1.55 \times 10^6$	Schmitz & Hof, 2005
Pyramidal cells (CA1-3)	Rat	STE	$615\text{-}930 \times 10^3$	Schmitz & Hof, 2005
Cerebral cortex	Rhesus monkey	STE	$1.35\text{-}2.95 \times 10^9$	Charvet et al., 2015
Cerebral cortex	Rhesus monkey	IF	0.825×10^9	Charvet et al., 2015
Purkinje cells	Rat	STE	$2.1\text{-}6.1 \times 10^5$	Herculano-Houzel et al., 2015
Lateral geniculate nucleus	Human	STE	$1.99\text{-}3.48 \times 10^6$	Herculano-Houzel et al., 2015
Neocortex	Human	IF	$12.7\text{-}16.3 \times 10^9$	Herculano-Houzel et al., 2015
Cerebellum	Human	IF	$54\text{-}65 \times 10^9$	Herculano-Houzel et al., 2015
Whole Brain	Human	IF	$67.3\text{-}86 \times 10^9$	Herculano-Houzel et al., 2015
Purkinje cells	Human	HIS	$0.88\text{-}26 \times 10^6$	von Bartheld et al., 2016
Purkinje cells	Human	STE	$15.4\text{-}30.5 \times 10^6$	von Bartheld et al., 2016
Dorsomedial thalamic nucleus (Schizophrenia)	Human	STE	0-40% loss	Dorph-Petersen & Lewis, 2017
Spiral ganglion (Young Adult)	Human	HIS	$23.1\text{-}33.7 \times 10^3$	Kaur et al., 2018
Spiral ganglion (Young Adult)	Human	STE	$26.7\text{-}41.7 \times 10^3$	Kaur et al., 2018

HIS, histology (profile counting = 2D Method); IF, isotropic fractionator; STE, stereology (= 3D Method)

Table 2

Calibration of counting methods against a serial section reconstruction ("gold standard") or against other counting methods

Structure	Embedding	Species	Method	Numerical Differences [*]	References
Against "Gold standard" (Exhaustive Counting)					
DRG	Paraffin	Rat	2D: Floderus	+39%	Coggeshall et al., 1990
DRG	Paraffin	Rat	2D: Abercrombie	+25.5	Coggeshall et al., 1990
DRG	Paraffin	Rat	2D: Konigsmark	+27.8%	Coggeshall et al., 1990
DRG	Paraffin	Rat	2D: Devor	+29.8%	Coggeshall et al., 1990
DRG	Paraffin	Rat	2D: Empirical	-0.5%	Coggeshall et al., 1990
DRG	Paraffin	Rat	2D: Rose & Rohrlich	-30.3%	Coggeshall et al., 1990
DRG	Paraffin	Rat	All 2D Methods	-30 to +39%	Coggeshall et al., 1990
DRG	Paraffin	Rat	Physical Disector	±8% to 12%	Pover & Coggeshall, 1991
DRG	Paraffin	Rat	2D: Empirical	-3.2% to +1%	Pover & Coggeshall, 1991
DRG	Paraffin	Frog	2D Method	-8% to -30%	St Wecker & Farel, 1994
DRG	Paraffin	Frog	Physical Disector	±4% to -26% [**]	Popken & Farel, 1996
Trochlear nucleus	Paraffin	Chicken	Optical Disector	-22.8%	Hatton & von Bartheld, 1999
Trochlear nucleus	Paraffin	Chicken	2D Method	-9.1%	Hatton & von Bartheld, 1999
Trochlear nucleus	Paraffin	Chicken	Modified Optical Disector	-1.0%	Hatton & von Bartheld, 1999
DRG	Paraffin	Frog	Physical Disector	±4% to ±26%	Farel, 2002
Glomerulus	Resin	Mouse	Physical Disector	-1.7% [-17 to +17]	Bagzen et al., 2006
Glomerulus	Resin	Mouse	2D Method	+10.2% [+1.2 to +20]	Bagzen et al., 2006
Trochlear nucleus	Various	Chicken	Optical Disector	-26% to +0.7%	Ward et al., 2008
Substantia nigra	Paraffin	Mouse	Optical Disector	+5%	Basquet et al., 2009
Substantia nigra	Paraffin	Mouse	2D Method	-4%	Basquet et al., 2009
DRG	Epon	Rat	Physical Disector	±6 to -27% [***]	Delaloye et al., 2009
IF Against Other Counting Methods					
White matter	Paraffin	Various	IF vs. Optical Disector	±0	Bahney & von Bartheld, 2014
Cerebral cortex	Frozen	Chimpanzee	IF vs. Optical Disector	+5%	Miller et al., 2014
Various	Frozen	Chicken	IF vs. Optical Disector	±0	Ngwenya et al., 2017

Abbreviations: DRG, dorsal root ganglion; IF, isotropic fractionator; 2D, 2D Method; [ ], ranges.

*
Note that numerical differences are composed of both, potential biases as well as statistical variability.

**
In this study, the orientation of sectioning appeared to be correlated with the extent of numerical differences, a finding that has no stereological explanation and does not seem to have been replicated.

***
the larger deviation was seen only when the separating distance between consecutive pairs of sections was larger than 60 μm.