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Research paper

The efficiency of design-based stereology in estimating spiral ganglion populations in mice

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ABSTRACT

Accurate quantification of cell populations is essential in assessing and evaluating neural survival and degeneration in experimental groups. Estimates obtained through traditional two-dimensional counting methods are heavily biased by the counting parameters in relation to the size and shape of the neurons to be counted, resulting in a large range of inaccurate counts. In contrast, counting every cell in a population can be extremely labor-intensive. The present study hypothesizes that design-based stereology provides estimates of the total number of cochlear spiral ganglion neurons (SGNs) in mice that are comparable to those obtained by other accurate cell-counting methods, such as a serial reconstruction, while being a more efficient method. SGNs are indispensable for relaying auditory information from hair cells to the auditory brainstem, and investigating factors affecting their degeneration provides insight into the physiological basis for the progression of hearing dysfunction. Stereological quantification techniques offer the benefits of efficient sampling that is independent of the size and shape of the SGNs. Population estimates of SGNs in cochleae from young C57 mice with normal-hearing and C57 mice with age-related hearing loss were obtained using the optical fractionator probe and traditional two-dimensional counting methods. The average estimated population of SGNs in normal-hearing mice was 7009, whereas the average estimated population in mice with age-related hearing loss was 5096. The estimated population of SGNs in normal-hearing mice fell within the range of values previously reported in the literature. The reduction in the SGN population in animals with age-related hearing loss was statistically significant. Stereological measurements required less time per section compared to two-dimensional methods while optimizing the amount of cochlear tissue analyzed. These findings demonstrate that design-based stereology provides a practical alternative to other counting methods such as the Abercrombie correction method, which has been shown to notably underestimate cell populations, and labor-intensive protocols that account for every cell individually.

1. Introduction

Comparing cell populations is an essential tool in assessing neural survival and degeneration in experimental groups. In order to evaluate the extent and significance of such survival and degeneration, accurate methods of quantification of neuronal populations are needed. Spiral ganglion neurons (SGNs) of the cochlea are indispensable in relaying auditory information from hair cells to the auditory brainstem, and studying their survival and degeneration provides insight into the physiological basis for the progression of hearing loss. Despite decades of research, techniques for quantifying the number of SGNs in a cochlea remains quite varied across studies (Richter et al., 2011).

Current cell counting techniques include three-dimensional serial reconstruction, two-dimensional cell body profile counts often with assumption-based correction factors, and stereological analysis. Serial reconstructions of three-dimensional tissue volumes most accurately determine population size but require an extremely labor-intensive process because they account for each cell individually. This method requires an observer to count every cell in the first section, then overlay the image of the second section and count all cells that do not appear in the first section. This process repeats until all cells have been accounted for and uses every section from the region under study. A three-dimensional view of the cochlea may also be constructed using specialized

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protocols with confocal microscopy and thin-sheet laser imaging microscopy (Johnson et al., 2011; MacDonald and Rubel, 2008, 2010). Two-dimensional profile counts vary widely depending on the parameters used, such as section thickness, and reflect estimates of cross-sectional profiles rather than of the actual population. These methods are particularly vulnerable to variation in neuron size and shape, and many studies report a raw count of SGNs without applying any sort of correction factor. However, even studies that employ assumption-based methods, usually two-dimensional profile counts with the assumption-based Abercrombie correction factor, which multiplies profile counts by a correction factor based on an assumed neuronal diameter, consistently underestimate SGN counts by 44%, possibly from variation in cell shape and orientation and uneven tissue shrinkage (Mouton, 2001; Guillery, 2002; Ishiyama et al., 2011). This problem can be compounded by changes in cell size and shape that may occur during neural degeneration, regeneration, and recovery. For instance, the addition of exogenous neurotrophins in response to hearing loss results in SGN survival and also an increase in SGN soma size, emphasizing the need for a counting method unaffected by such changes (Glueckert et al., 2008; Shepherd et al., 2008).

Stereological methods are the least prevalent approach but may offer an efficient and accurate alternative. Design-based stereology, also called unbiased stereology, is an analytical tool that is used to quantify populations of naturally occurring three-dimensional objects based on their two-dimensional cross sections. Unlike biased counting methods, estimates obtained by stereological methods are not significantly affected by the counting parameters used or the size and shape of the cell and increased sampling improves precision rather than further confounding the data. In order to sample efficiently without sacrificing accuracy, stereology must avoid double-counting objects in the small areas under evaluation. It minimizes this error in a two-dimensional plane by setting a standardized counting frame around each counting site and in the third dimension by comparing two such parallel planes, whether optical or physical. The physical disector and the optical disector are two methods that apply these stereological principles to cell counting. The physical disector relies on precisely aligning images of two adjacent serial sections and ensuring that all cells are counted once, without double counting the cells that appear in both sections. Although the process is similar to a serial reconstruction, the physical disector only requires a sample of pairs of adjacent sections, rather than all sections from the region of interest. The optical disector, used in the present study, removes the need for adjacent sections since the observer scrolls between two optical planes within a single thick section and marks each cell that comes into focus. During this analysis, measurements are taken that enable observers to detect tissue shrinkage or deformation and appropriately accommodate such irregularities to minimize bias (Dorph-Petersen et al., 2001).

Ideally, researchers should adopt a method of quantifying SGNs that strikes a balance between practicality and accuracy. Certain methods will be most practical for a particular study. Counting all neurons within Rosenthal's canal using unbiased techniques will undoubtedly result in the most accurate data (Richter et al., 2011). The total number of SGNs is often not the only measure of interest, but rather one of a number of anatomical and functional measures within a study. For instance, thick osmicated cross sections through the cochlea may be collected with the intention of selecting small pieces of tissue for electron microscopic evaluation (e.g., Lauer et al., 2012; Stamataki et al., 2006) or evaluating the status of both neural and non-neural cochlear structures in a mutant mouse model (e.g., Hequembourg and Liberman, 2001; Ohlemiller and Gagnon, 2007). In such cases, counting every neuron is not necessarily practical or necessary. The present study aims to demonstrate that design-based stereology accurately estimates the total number of cochlear SGNs in mice using assumption-free methods, while being more efficient than a serial reconstruction. This study also suggests that design-based stereology is sensitive to hearing loss-related SGN degeneration by comparing stereological SGN counts in young normal-hearing C57Bl/6J (C57) mice to counts from old C57 mice, a strain that has a well-characterized genetic predisposition to age-related hearing loss and cochlear degeneration (Mikaelian, 1979; Li and Borg, 1991; Francis et al., 2003; Hequembourg and Liberman, 2001). This technique is applicable to other animal models as well.

The optical disector stereological probe calculates an estimate by counting cells from a fraction of the sections spanning a region of interest. Objects that come into focus between the top and bottom optical planes of that section are counted, and varying the section thickness or section fraction does not affect final population estimate, as it would in profile counting. This reduces the number of sections required for analysis compared to the serial reconstruction method that requires analysis of every section. Some recent studies have opted to compare two-dimensional SGN densities; however, this cannot be used to estimate the total population. We describe a procedure for using the optical fractionator probe and compare it to other methods of estimating SGN populations in mice.

2. Materials and methods

2.1. Subjects

Adult C57 mice were anesthetized with ketamine and xylazine in ethanol and perfused intracochlearly with 1% osmium tetroxide in 1% potassium ferricyanide, decalcified, dehydrated in graded alcohols, and embedded in Araldite as part of previous experiments (Francis et al., 2003; Stamataki et al., 2006). The cochleae were sectioned at 40 μm parallel to the modiolus, then mounted between sheets of Aclar and analyzed using the procedures described below. The reader is referred to those papers for more detailed descriptions of tissue harvest and preparation techniques. Four cochleae were analyzed from young mice ages 8–11 weeks old, and four cochleae were analyzed from mice 8–11.5 months old. All subjects appeared healthy, with normal respiratory activity, normal tympanic membranes, and with no evidence of external or middle ear infection at the time of tissue harvest. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and with the approval of the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

2.2. Stereology

Though we performed the stereological analysis on osmicated tissue samples, the basic procedures described here can be applied to cochlear sections prepared using other neuron stains such as hematoxylin–eosin, toluidine blue, or with unstained tissue visualized using differential interference contrast (Nomarski) or phase contrast microscopy. SGNs were counted using the Optical Fractionator Probe in the StereoInvestigator (SI) software (Microbrightfield Bioscience, Williston, VT) using parameters determined by a pilot study and based on those described by Camarero et al. (2001). The reader is referred to the online Optical Fractionator Probe workflow webinar available for download at www.mbfbioscience.com for a tutorial explaining how to use the software. Similar procedures may be used with the Optical Fractionator probe included with other commercially available stereology systems such as Stereologer. The area sampling fraction (asf) was calculated from a sampling grid of 120 × 120 μm and counting