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Estimation of the number of nerve fibers in the human vestibular endorgans using unbiased stereology and immunohistochemistry

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Abstract

The objective of this study was to obtain estimates of the number of nerve fibers in the human crista ampullaris and utricular macula from normal individuals using unbiased stereology and immunohistochemistry. Vestibular endorgans with the attached vestibular nerve stump were microdissected from the temporal bones. Specimens were divided into two groups. The first group (group 1, $N = 8$, age range, 68–98 years old, mean = 87 years) was fixed with paraformaldehyde and post-fixed with osmium tetroxide. The second group (group 2, $N = 5$, age range, 80–98 years old, mean = 86.6 years) was fixed with paraformaldehyde, immunoreacted with monoclonal antibodies against neurofilaments, and post-fixed with osmium tetroxide. The endorgans of both groups were embedded in resin and 2-μm thick sections were made. Estimates of the number of nerve fibers were obtained using an unbiased stereological method, the fractionator. The diameter distribution of nerve fibers was also obtained. The average number of fibers in the horizontal, posterior and superior cristae of individuals in group 1 ($N = 14$ cristae) was 1424 ± 320 (CV = 0.22). The average percentage of small (less than 3 μm), medium (between 3 and 5 μm) and large (more than 5 μm) size fibers was 22.4%, 51.5% and 26.1%, respectively. In group 2 ($N = 12$), there was an average of 1792 ± 99 (CV = 0.05) nerve fibers. The average percentage of small, medium and large size fibers was 22%, 51.2% and 26.8%. In the macula utricle from group 1, there was an average of 3026 nerve fibers ($N = 2$, ages 80 and 96 years old). There was an average 30.75% small, 56% medium and 13.2% large size fibers. In the utricular macula from group 2 ($N = 3$, ages 84, 92 and 96 years old), there was an average of 3715 nerve fibers. The average percentage of small, medium and large size fibers was 33.2%, 51.7% and 15.1%. The nerve fiber number in both groups is within the range of previous studies, however, the number of fibers in group 2 was significantly higher than that in group 1 ($p = 0.01$). This difference is likely due to increased sensitivity gained by the immunohistochemical staining of the axoplasm of nerve fibers in group 2. Results from the present study demonstrate the use of unbiased stereology and immunohistochemistry in human vestibular endorgans, as a reliable and efficient method to estimate the number of nerve fibers. These methods can be applied for studies of normal aging and pathological conditions of the vestibular periphery.

Keywords: Vestibule; Microdissection; Nerve fibers; Stereology; Immunohistochemistry; Human temporal bone; Inner ear; Vestibular periphery

1. Introduction

In the mammalian vestibular sensory neuroepithelium, there are two types of hair cell receptors. The flask-shaped type I hair cells are surrounded by a calyceal afferent terminal. The cylindrical type II, hair cells are innervated by bouton terminals (Fernandez et al., 1988, 1995; Lysakowski and Goldberg, 1997; Goldberg et al., 1992; Goldberg, 2000). The morpho-physiological and ultrastructural characteristics of the afferent innervation in the mammalian vestibular endorgans have been described in detail in the squirrel monkey, chinchilla and gerbil (Fernandez et al., 1990, 1995; Goldberg et al., 1990; Lysakowski and Goldberg, 1997;

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Purcell and Perachio, 2001). In the mammalian crista, there are three types of afferent vestibular nerve fibers that innervate type I and type II hair cells (Fernandez et al., 1988, 1990; Goldberg et al., 1990). One group of afferent neurons with the largest fibers, the calyceal afferents, corresponding to about 10% of total in the chinchilla model, innervate exclusively one or several type I hair cells by way of a single or 2–4 complex calyces found in the cuspid of the mid-portion of the crista (central zone). A second group of afferent neurons with thinner fibers, corresponding to about 20% of total, the bouton afferents, innervate many type II hair cells with multiple bouton terminals, in the peripheral margin of the crista (peripheral zone). A third group of afferent neurons with medium size fibers, the most numerous at about 70% of total, the dimorphics, innervate both type I hair cells with calyces and adjacent type II hair cells with bouton-bearing short collateral branches throughout the whole crista (Fernandez et al., 1988; Lysakowski and Goldberg, 1997). The classification of utricular afferents by the peripheral innervation pattern is similar to the crista ampullaris: calyceal, dimorphic and bouton afferents (Fernandez et al., 1990). However, the relative proportions differ: calyceal units account for 1.3%, dimorphic units 86.4% and bouton units 12.2%. The dimorphic units are further subclassified into the striolar (the central portion of the utricular macula) accounting for 3.7% and extrastriolar 82.7% (Fernandez et al., 1990).

The number of fibers in the vestibular nerve and its individual branches have been determined in man, squirrel monkey and chinchilla using traditional quantitative techniques (Rasmussen, 1940; Bergstrom, 1973a,b,c; Honrubia et al., 1987; Lee et al., 1990; Naito et al., 1997). The techniques used in previous studies were either assumption-based methods, or the quantification of each nerve fiber within horizontal sections using reconstruction and camera lucid drawings. While the counting of each nerve fiber is an accurate method, it is time consuming. Unbiased stereology is the gold standard to obtain morphometric estimates of three-dimensional objects from the two-dimensional sections through a specimen. Unbiased stereological techniques sample the region of interest uniformly and count objects (e.g., cells, nucleoli and synapses) without relying on assumptions about size, shape and orientation of the objects (Gundersen et al., 1988; Tang et al., 2002).

Unbiased stereological methods have been applied for quantitative analysis in the mammalian inner ear (Baurle and Guldin, 1998; Desai et al., 2005a, 2005b; Fernandez et al., 1995; Ishiyama et al., 2004; Lysakowski and Goldberg, 1997; Lopez et al., 1997; Merchant, 1999; Merchant et al., 2000; Severinsen et al., 2003). We have recently developed a stereological approach for use with archival temporal bone specimens (Park et al., 2000, 2001; Tang et al., 2002) and applied it to obtain estimates of the number of hair cells in the utricular macula and the number of vestibular ganglion cells in microdisected end organs and Scarpa's ganglia obtained from post-mortem human temporal bones (Gopen et al., 2003; Ishiyama et al., 2004). We utilized the microdissection technique (Lars-Goran and Hawkins, 1972; Lee et al., 1990; Wright and Meyerhoff, 1989), in which the cochlea, three cristae ampullaris and utricular and sacular maculae are dissected from the otic capsule of the temporal bone, and endorgans are individually post-fixed with osmium tetroxide. Osmium tetroxide allows for the preservation, visualization and quantification of myelinated fibers (Lee et al., 1990; Rosenhall, 1972a,b). However, the identification of unmyelinated fibers is not possible with this method. In cases where myelin loss may occur due to aging or other pathological conditions, the quantification of nerve fibers using traditional staining techniques may not be accurate.

The identification of nerve fibers in vestibulo-cochlear endorgans can be made with the use of specific antibodies against neurofilaments and immunohistochemistry (Dau and Wenthold, 1985; Hafidi and Romand, 1989; Hsu et al., 1997; Nishizaki and Anniko, 1995). Neurofilament proteins are intermediate filaments that are specific to neurons, dendrites and axons. Neurofilament subunit immunoreactivity has been used successfully to identify the axoplasm of myelinated and unmyelinated fibers in the vestibulo-auditory nerve (Dau and Wenthold, 1985; Hafidi and Romand, 1989). Additionally, the expression of neurofilaments may be altered by conditions such as development, aging, degeneration and regeneration of neurons (Dau and Wenthold, 1985; Zaffaroni, 2003). Recently, three different fibers size subtypes have been identified with the use of specific antibodies against peripherin (thin bouton afferents), calbindin (medium dimorphic afferents) and calretinin (thick calyceal afferents) (Desai et al., 2005a, 2005b; Dememes et al., 1992; Desmadryl and Dechesne, 1992; Leonard and Keveter, 2002; Lysakowski et al., 1999). The combination of immunohistochemical and histological techniques allows the visualization of myelinated and unmyelinated nerve fibers of different subtypes.

The objective of this study was to estimate the number of nerve fibers in the microdissected human semicircular canal cristae and macula utricle from normal individuals using unbiased stereological techniques. The axoplasm of nerve fibers and terminals located in the crista or utricle stroma were labeled with specific monoclonal antibodies against neurofilament proteins (pan neurofilament antibody that recognizes three types of neurofilament proteins: 68, 160, and 200 kDa) along with traditional osmium tetroxide post-fixation to stain myelin. This report demonstrates the advantage of the combination of quantitative unbiased stereological and immunohistochemical techniques over single method approaches to identify and quantify vestibular nerve fibers in the human cristae ampullaris and macula utricle.

2. Methods

2.1. Human tissue

Temporal bones were obtained from 9 patients with well-documented clinical records, no history of auditory or