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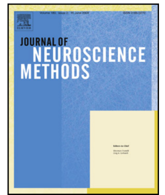

Basic Neuroscience

Comparison of unbiased estimation of neuronal number in the rat hippocampus with different staining methods

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HIGHLIGHTS

- The accuracy of neuronal number in hippocampus is particularly important in neuropathological processes and treatments.
- The stereological method offers 3-D unbiased estimation for the total number of neurons in a defined brain region.
- We provided a reliable and feasible method for quantification of total neuronal numbers by using unbiased stereological estimation.

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ABSTRACT

Background: NeuN and Nissl staining (toluidine blue, cresyl violet staining) are routinely used methods in unbiased stereological estimation of the total number of hippocampal neurons.
New method: In the present study, we stained serial frozen coronal sections from 5 normal adult male Sprague-Dawley rat brains with different methods, measured the deformation of hippocampal area in brain sections and estimated the total number of hippocampal neurons using the optical fractionator.
Results: The deformation in x, y-axis was not obviously different with different staining protocols, but shrinkage in z-axis was significant after staining ($p < 0.001$). NeuN staining produced significant higher estimate number than cresyl violet staining by 24% ($p = 0.002$), however, NeuN and Cresyl Violet staining showed a high degree of correlation in quantification of total neuronal numbers and both methods are suitable for unbiased stereological estimation.
Comparison with existing method (s): NeuN is more reliable but if time is limited or the number of animals used in experiments is high, cresyl violet staining may be a feasible method.
Conclusions: Compared with previous estimates of the neurons number in rat hippocampus, our present data is reliable and the stereological analysis based on our system is a cost-effective unbiased method for estimation of neuron number.

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1. Introduction

The accurate quantification of neurons is critically important not only to the understanding of physiological and psychological performance in some neuropathological processes, such as traumatic brain injury (TBI) (Anderson et al., 2005; Grady et al., 2003; Maxwell et al., 2003), Parkinson's disease (PD) (Ma et al., 1997), Alzheimer's disease (AD) (Gómez-Isla et al., 1996; Zarow et al., 2003) and Amyotrophic Lateral Sclerosis (ALS) (Gredal et al., 2000; Li et al., 2015), but also to the screening of neuroprotective drugs and evaluating the efficiency of treatment strategies in pre-clinical studies (Li et al., 2015; Zhu et al., 2015). There are several counting methods used in the field of neuroscience research, such as flow cytometry (Collins et al., 2010; Young et al., 2012), manual counting (Neubauer chamber) (Bertucco et al., 1998; Collins et al., 2010), automatic or semi-automatic counting methods (Oberlaender et al., 2009; Woeffler-Maucler et al., 2014), and unbiased stereological methods (Heggland et al., 2015; Kempermann et al., 1997). The stereology was established by G. Buffon in 1733 (Miyamoto, 1994), which is based on statistical sampling principle and Cavalieri's principle (fundamental principles of geometry). Stereological design-based method is widely used in neuroscience and which can

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offer unbiased, accurate and precise estimates of neuronal number in a defined brain region (Kawagishi et al., 2014; West et al., 1991). The optical fractionator is a three dimensional probe used in stereology for number counting, it is very efficient and the estimate requires relatively thick sections (≥20 μm after processing and cover slipping) (Wirenfeldt et al., 2003). Systematic random sampling (SRS) is used in the optical fractionator method to obtain unbiased and quantitative data. In recent years, a debate about appropriate methods of cell counting in tissue sections has arisen, and the weaknesses and strengths of 3-D versus 2-D counting approaches were reviewed and compared by some investigators (Benes and Lange, 2001; von Bartheld, 2001). But for both the 3-D or 2-D counting methods, calibration and validation are needed because we should know what could generate the biases, e.g. the size of the counting frame, inclusion criteria for recognition of neurons, ratio of shrinkage of sections, optical fractionator height, staining quality, etc. (von Bartheld, 2001). Anti-NeuN, Toluidine blue and Cresyl violet staining are very common histological staining methods used in stereology, morphological and neurobiological studies. NeuN is a neuronal specific nuclear protein (Mullen et al., 1992), has been used as a neuronal marker in an increasing number of brain studies (Blaya et al., 2014; Lu et al., 2007; Lu et al., 2002). Toluidine blue and cresyl violet staining are also used in neurobiological studies (Aungst et al., 2014; Calhoun et al., 1998; Kulesza Jr et al., 2002; Lowenstein et al., 1992) although both of methods are not specific stain for neurons.

In the present study, we used the optical fractionator method, and optimized our tissue processing and three staining protocols for neuronal visualization by NeuN immunohistochemistry, Toluidine blue and Cresyl violet staining. We evaluated the deformation of brain tissue after staining along *x*, *y*-axis and *z*-axis, respectively. The total number of neurons in hippocampus was estimated following a set of principles after pilot counting. We calibrated and validated the systematic uniform sampling scheme by three different staining methods. We aimed to establish a low time consuming, reliable, and repeatable unbiased number counting method.

2. Materials and methods

2.1. Animals

Five healthy adult male Sprague-Dawley rats (375–400 g) were used in the present study. The animals were housed with a 12/12 light/dark cycle and had free access to food and water ad libitum for at least one week before experiments according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC), The Ohio State University.

2.2. Perfusion and section

All animals were deeply anesthetized with 1.5% isoflurane, the heart was rapidly exposed. A catheter was introduced into the ascending aorta and the right atrium was incised. 200 ml of saline (1000 IU of heparin in saline) was perfused through the catheter at a rate of 25 ml/min followed by 400–500 ml of cold 4% paraformaldehyde (PFA, PH = 7.4) in phosphate buffered saline (PBS). The brains were removed carefully, post-fixed in 4% PFA (PH = 7.4) overnight at 4 °C, and then cryoprotected with 30% sucrose for 72 h. Serial frozen coronal sections (50 μm thick) were cut using a cryostat (Microm HM 525, Thermo scientific) at −18 °C, all sections containing the hippocampus were gathered in order and kept in 24 wells plates containing 4% PFA (PH = 7.4) and stored at 4 °C. The sections were then processed for immunohistochemical staining, Toluidine blue staining and Cresyl violet staining.

Table 1
The sampling scheme used in present study.

	NeuN immunostaining	Toluidine blue staining	Cresyl violet staining
1/ssf	1/12	1/12	1/12
h (μm)	14.88	19.15	16.32
t (μm)	14.88	19.15	16.32
a (frame) (μm²)		40 × 40 = 1600	
A (*x*, *y* step) (μm²)		900 × 900 = 810,000	
1/asf = *A*/*a*		900 × 900/(40 × 40) = 506.25	

2.3. Immunohistochemistry

Immunohistochemical staining was carried out as follows: free-floating sections were rinsed in 0.1 M PBS (PH = 7.4) 3 times and incubated in 3% H_2O_2 in methanol for 10 min and then blocked for nonspecific antigen binding using 4% BSA/0.1% Triton-100/PBS (BP+) for 1 h at room temperature. This was followed by an overnight incubation with primary antibody (1:2000 in PBS, mouse anti-NeuN, Chemicon, MAB377) at 4 °C. Sections were then rinsed in 0.1 M PBS 3 times and incubated with biotinylated horse anti-mouse IgG (1:500 in PBS, Vector Laboratories, BA-2001) for 1 h at room temperature, and then the sections were treated with Elite avidin-biotin enzyme complex (ABC; Vector Laboratories) for 1 h. Visualization of labeling was achieved using 3,3′-diaminobenzidine (DAB, Vector Laboratories, SK-4100). Sections were rinsed, dehydrated in 95%, 100% ethyl alcohol, cleared in xylene and coverslipped with Permount (Fisher Scientific) in a fume hood.

2.4. Toluidine blue staining

The slide-mounted brain sections were stained in 0.03% Toluidine Blue solution (PH = 1.5) for 1–2 min after dry in air, rinsed with dH_2O, then decolorized in 75% ethyl alcohol and dehydrated in 95%, 100% ethyl alcohol for 2 to 3 min. The sections were then cleared in xylene for 2 to 3 min and mounted with Permount (Fisher Scientific) in a fume hood.

2.5. Cresyl violet staining

The slide-mounted brain sections were differentiated in 95% ethyl alcohol at least 5 h at room temperature before staining, rinsed in 75% ethyl alcohol 5 min and in distilled water 5 min. Next, the sections were stained in 0.1% cresyl violet (Sigma) solution for 2–3 min and then rinsed quickly in distilled water. After decolorization in 75% ethyl alcohol for few seconds, the sections were dehydrated in 95%, 100% ethyl alcohol for 2 to 3 min, cleared in xylene for 2 to 3 min and mounted with Permount (Fisher Scientific) in a fume hood.

2.6. Unbiased estimation of the hippocampal neurons number

Total neuronal cell number in hippocampus of rat was estimated and calculated using the Cavalieri principle. In our study, NIS-Elements AR software (Version 4.20.00, Nikon, Japan) and Nikon ECLIPSE Ci-L microscope system (Nikon, Japan) was used. The microscope was equipped with a motorized microscope X, Y stage and Z stage (H101A/B, Prior Proscan III). The built-in XYZ navigation function of the software provided high-precise control of movement along *X*, *Y* and *Z* axes. The live image stream generated with Nikon Digital Sight DS-U3/DS-Ri1 (Nikon, Japan) was transferred to a monitor (Dell) connected with a computer (Z400 workstation, HP) and a 2 × objective lens (N.A. = 0.06, W.D. = 7.5 mm) and a 100 × oil-immersion lens (N.A. = 1.300, W.D. = 0.16 mm) were used for the unbiased counting and analysis.

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