

A COMPARISON OF 2D IMAGE ANALYSIS AND DESIGN-BASED STEREOLOGY
FOR EVALUATING MORPHOLOGICAL AND ANATOMICAL CHANGES
IN THE DOPAMINERGIC SYSTEM OF THE RODENT MIDBRAIN

BY

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Appendices:

Appendix A: Comprehensive Literature Review I: What is the relationship of anatomical structures and functions of the substantia nigra and the ventral tegmental area?

Appendix B: Comprehensive Literature Review II: What is the potential of animal models to inform occupational theory theories and treatment?

Appendix C: Comprehensive Literature Review III: How does stereology help to inform translation from neuroscience to occupational therapy?

Abstract

Background. 2D analyses produce systematic errors in quantifying anatomical and morphological features in the brain. Design-based stereology overcomes this limitation by applying probability theory, yet many neuroscience investigators still use 2D analyses. The purpose of this study is to compare 2D analysis with design-based stereology in quantifying differences of morphological and anatomical features between groups.

Methods. Brain tissue samples of three different rodent models were analyzed; chronic MPTP/probenecid PD (MPD) mouse model, alcohol preferring (AP) rat model, and the enriched environment (EE) rat model. 2D analyses and design-based stereology were used to quantify neuronal number, neuronal volume and regional volume. Student's *t*-test (two-tailed) was used to compare quantitative data.

Results. 2D analyses generated significantly different estimation from design-based stereology in neuronal number and did not find relatively small differences of neuronal number. 2D analysis generated comparable value to design-based stereology in normalized data but not in actual value. 2D estimated accurately regional volume.

Discussion. 2D analyses may be used for rough screening to find a difference of neuronal number and volume but should not for the estimation of actual value. Design-based stereology should be used to estimate neuronal number and volume. Both 2D analyses and design based stereology can be used for the estimation of regional volume.

Introduction

In neuroscience, major light microscopic morphometric analyses fall into two major camps, two-dimensional (2D) image analysis and three-dimensional (3D) analysis. (Benes & Lange, 2001; Guillery & Herrup, 1997; Selemon & Rajkowska, 2002; von Bartheld, 2001) Both types of quantitative analyses have been used to quantify the number of neurons, the size of neurons, and the size of regions in the brain. (Beasley, Honavar, Everall, & Cotter, 2009; Coggeshall & Lekan, 1996; Metz, Antonow-Schlorke, & Witte, 2005) 2D image analyses quantify these parameters derived from cross-sectional profiles with methodological assumptions in the form of mathematical correction factors. (Coggeshall & Lekan, 1996; Lu, Chen, & Poon, 2009) Because of these assumptions, 2D image analyses display systematic error in the estimation of morphological and anatomical features in the brain. (Sterio, 1984; West & Gundersen, 1990) Design-based stereology overcame systematic error of 2D analyses by applying probability theory within a 3D sample frame. (Schmitz & Hof, 2005) Design-based stereology (3D) is widely accepted as the gold standard of light microscopic morphometric analyses in the brain. (Benes & Lange, 2001; West, 1999) Regardless of systematic error, 2D morphometric analyses are commonly used to demonstrate morphological and anatomical features of the brain such as neuronal number, neuronal density, neuronal volume, total volume and lesion size. (S. O. Ahmad, Park, Stenho-Bittel, & Lau, 2009; Khan, et al., 2009; Petzinger, et al., 2007) In morphometry, there are three basic 2D image analysis measures to quantify the number of cells in the brain; densitometry, the fractionator method and model-based stereology.

Three basic 2D analyses are used extensively to quantify the amount of objects in the brain. Densitometry is a quantitative analysis of optical density in light sensitive materials such as film or a thin tissue sample. Optical density is the ratio between dark and bright areas. Densitometry has been used to investigate the treatment effects in a rat model of Parkinson's

disease.(Marti, et al., 2005; Xue, Zhao, Guo, & Duan, 2007) The density of dopaminergic (DA) fiber in the striatum was measured as treatment effects by densitometry.(Xue, et al., 2007) In addition to densitometry, The fractionator method and model-based stereology are 2D methods to estimate the total number of cells in an area of interest.(Guillery & Herrup, 1997) The total number of counted cells serves as a substitute for the actual total number of cells in the area of interest.(Anastasia, Torre, de Erausquin, & Masco, 2009; Fujishiro, et al., 2006; Schintu, et al., 2009) In the 2D counting method, sample sections are selected randomly and all visible cells (full nuclei visible) are counted in the area of interest of the sample section. In the fractionator method, the total number of cells is estimated by counting cells in every n th section and then multiplying by n .(Brown, Gouty, Iyer, Rosenberger, & Cox, 2006; Guillery & Herrup, 1997) With this correction equation, the total number of tyrosine hydroxylase (TH) positive neurons in the SNpc was estimated in the rat model of PD.(Brown, et al., 2006) The fractionator method is thought to overestimate the total number of cells because cells cut in sectioning appear in more than one section.(Coggeshall & Lekan, 1996; Guillery & Herrup, 1997) Model-based stereology corrects this overestimation error by applying Abercrombie's correction factor.(Abercrombie & Johnson, 1946; Faherty, Raviie Shepherd, Herasimtschuk, & Smeyne, 2005; Hamre, Tharp, Poon, Xiong, & Smeyne, 1999; Rinne, Ma, Lee, Collan, & Roytta, 2008; Zhou, Zhang, Lumeng, & Li, 1995)

In addition to counting objects, 2D image analysis can quantify the size of cells and/or a specific brain area. The pixel counts of cells by 2D image analysis are used to compare cellular sizes as a substitute for the actual cell volume. In the research on dementia with Lewy bodies (DLB), sizes of ChAT-positive neurons were 600 pixels/cell in control and 400 pixels/cell in DLB.(Fujishiro, et al., 2006) The pixel counts are dependent on the magnification of the microscope and resolution of the screen. These pixel counts can be

calculated into actual area measurement by calibration of distance in the picture. In addition, 2D image analysis can estimate volumetric size of cells in the brain. The formula for the volume of a sphere ($\text{volume} = 4\pi r^3/3$) is used to estimate cellular volume based on 2D data.(Lu, et al., 2009) For this formula, the radius of the neuron can be estimated from the largest diameter or the area of neuronal profile.(Cooper & Sofroniew, 1996; Hamm, Temple, O'Dell, Pike, & Lyeth, 1996; Lu, et al., 2009) The volume of specific brain areas such as the area of lesion has been estimated also by 2D analyses.(Knieling, Metz, Antonow-Schlorke, & Witte, 2009; Millerot-Serrurot, et al., 2007) Model-based stereology quantifies specific brain area based on the measurement of cross-sectional area and the distance between sections. This 2D volume estimation is also used to investigate the therapeutic effect of enriched environment (EE) on cerebral ischemia in a rat model.(Knieling, et al., 2009) 2D image analyses have been used to quantify morphological and anatomical features such as the number of neurons, the volume of neurons, and the volume of a brain region.(Brown, et al., 2006; Coggeshall & Lekan, 1996; Cooper & Sofroniew, 1996; Faherty, et al., 2005; Fujishiro, et al., 2006; Rinne, et al., 2008) However, this method has some theoretical weaknesses because it relies on mathematical assumptions. 2D analyses assume that every cell has an equal chance to be counted in sample sections and that cells would be perfectly spherical in shape. This tends not to be the case as most neurons are not circular but irregular. Each part of a 3D object does not have an equal chance to be selected by cross sectioning in thin sections of the biological tissue, such as neurons, are not a classical shape. These sources of bias cause systematic error in 2D image analysis when counting cells and to estimating the size of cells.(West, 1999) Some of these disadvantages have led to the development of design-based stereology.

Design-based stereology (3D) has eliminated several theoretical obstacles to allow

increased precision in estimates of the 3D parameters derived from 2D cross-sections of biological tissues.(Schmitz & Hof, 2005) Design-based stereology offers methodological advantages for neuroscience research by applying probability theory to the estimation of parameters with a 3D sample frame.(Guillery & Herrup, 1997; Schmitz & Hof, 2005; Sterio, 1984; West & Gundersen, 1990) In counting objects, every object has the same chance to be counted by the optical disector principle.(Sterio, 1984; West & Gundersen, 1990) The nucleator method is used to estimate local volumes and the Cavalieri-point counting method is used to estimate the regional volume of biological area.(Schmitz & Hof, 2005) Any assumption is not needed to estimate cell volume and regional volume using the nucleator and the Cavalieri-point methods. The result is an increase in accurate estimation on all neuronal number, neuronal volume, and regional volume. This methodological development of design-based stereology tests previous studies and provides new implications. For example, Design-based stereology challenged previous beliefs derived from 2D analyses in that there is a progressive neuronal loss with normal aging. Until late 1980s, it was widely accepted that normal aging induced extensive neuronal loss in the brain especially in the hippocampus.(Coleman & Flood, 1987; Morrison & Hof, 1997) These studies used neuronal density, not total number, to quantify the number of neurons by 2D analysis.(Brody, 1955) Design-based stereology reexamined this view of significant neuronal loss with normal aging in 1990s.(Morrison & Hof, 1997) Several stereological studies have led to the innovative conclusion that age-related neuronal loss through apoptosis is not associated with normal aging in the hippocampus and neocortex.(Gomez-Isla, et al., 1996; Pakkenberg & Gundersen, 1997; West & Gundersen, 1990)

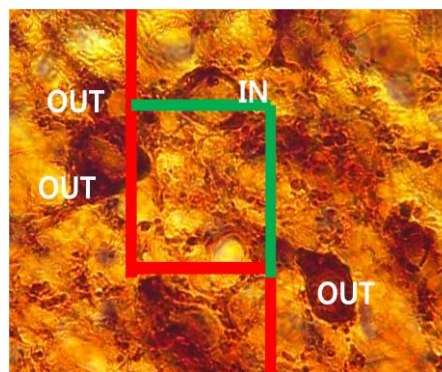
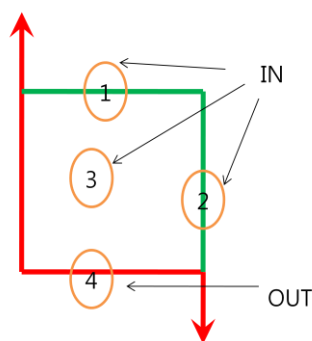
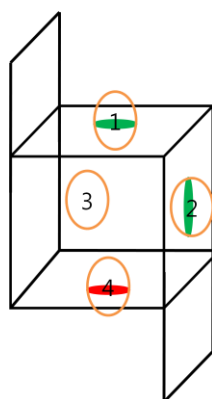
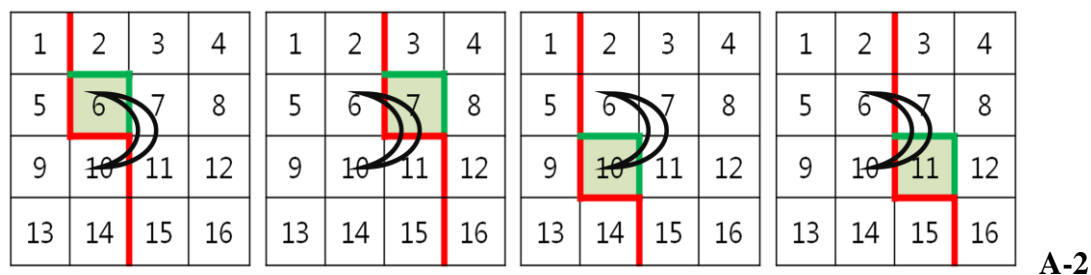
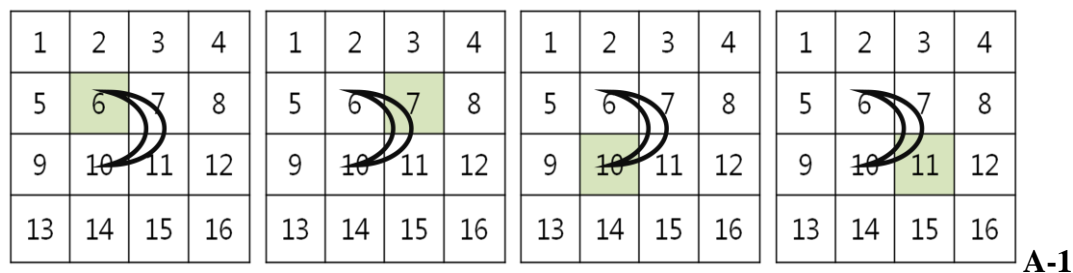


Figure 1. A. Edge bias; The object is counted in biased frames 6, 7, 10, and 11(A-1). The object, however, is counted in unbiased frame 10 only(A-2), **B. 3D unbiased probe;** Unbiased disector counting frames showing inclusion(green) and exclusion(red) line(2-D) and inclusion and exclusion(red) planes (3-D) (B-1). **C. Optical disector principle;** the neurons must be counted only appears for the first time within the unbiased count frame while the investigator optically section from top to the bottom in the z-axis.

Design-based stereology provides unbiased counting method with the disector principle. The disector principle avoids edge effects by using an unbiased frame and counting rules.(H. J. G. Gundersen, 1977; West, 1993) The edge problem arises when an object in the edge of the probe is counted more than one time using a biased frame (Figure 1, A).(Mandarim-de-Lacerda, 2003; West, 1993) The 3D disector probe consists of an inclusion plane and an exclusion plane. On the disector plane, the upper and right plane is the inclusion

plane while the lower and left plane is the exclusion plane (Figure 1, B). The exclusion plane extends to infinity above the left plane and below of the right plane. In addition to these planes, the top and bottom of the 3D probe are covered with a guard volume, which avoids an artificial effect created from cutting the section with blade. This is called “capping” or cutting the neurons in half. Using unbiased counting rules, the objects within the 3D probe or touching the inclusion bars must be counted, while the object out of the 3D probe or touching the exclusion bars must not be counted. Based on this counting method with the disector principle, the total number of objects is calculated by the following equation:

$$E(N) = \sum Q \cdot F_1 \cdot F_2 \cdot F_3$$

$E(N)$ = estimate of total number of object; $\sum Q$ = Sum of objects counted using disector counting rules; F_1 = 1/section sampling fraction = 1/(number of sections sampled/total sections); F_2 = 1/area sampling fraction = 1/(area of section sampled/total area); F_3 = 1/thickness sampling fraction = 1/(disector height/section thickness)

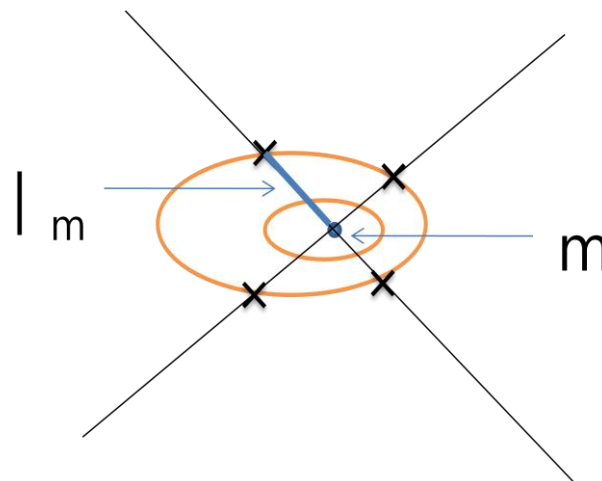


Figure 2. The nucleator method; m ; the center of the cell, l_m ; the line length between the center and the border of the cell.

In design-based stereology, the nucleator method is used to estimate local volumes such as cellular or nuclear volumes of an individual neuron. The main idea of the nucleator principle is that a mean length of lines randomly oriented across profiles of a population of objects is used for the estimation of the mean volume of the objects. After the center of cell

(m) is determined, the line length, l_m , between the center (nucleus) and the border of the object's profile are measured in a random direction. If more than one random line is applied on the profile, the mean line length from the separate line lengths are used in following formula for the volume estimate.

$$\text{Mean cell volume} = \text{mean } l_m^3 (4\pi/3)$$

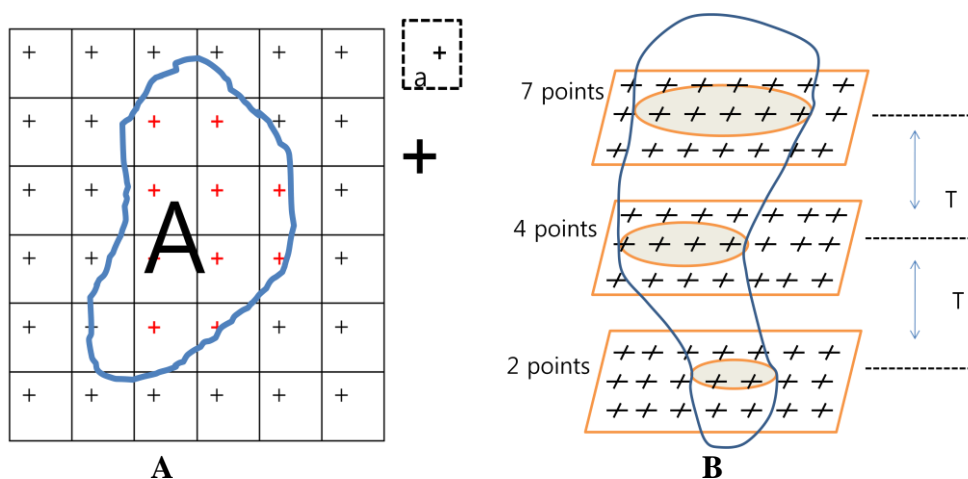


Figure 3. The Cavalieri-point counting method to estimate volume; A. Counting the sum of dimensionless points (arrow) spaced apart by area, a , and placed at random over profiles leads to unbiased estimates of the expected profile area. **B.** Combination of the Cavalieri volume and point counting to estimate total volume of an arbitrarily shaped object.

Design-based stereology estimates total volume of an object by the Cavalieri-point method. The Cavalieri-point counting method is used to estimate the volume of arbitrary shaped biological objects. (Kubínova & Janáček, 2001) Bonaventura Cavalieri demonstrated that the volume of arbitrary shaped objects can be estimated with the sum of the area selected randomly throughout the whole object and the distance between selected planes. (Mandarin-de-Lacerda, 2003) Based on the Cavalieri method, the Cavalieri-point counting was developed in 1930. (Thomson, 1930) Instead of the profile area, this new technique uses the number of selected points within the profile area. Points are placed in a systematic-random manner throughout the reference area as well as in individual sections. The formula to estimate volume of object K is

$$\text{Volume } K = T \times \Sigma P \times a(p)$$

Volume K = regional volume of the object; T = the section interval distance; P = total number of point hitting the reference area in each section; a (p) = the area per point.

Reliable quantitative analysis methods are critical in investigating morphological and anatomical changes in the brain.(Baquet, Williams, Brody, & Smeyne, 2009; Coggeshall & Lekan, 1996) From a theoretical perspective, design-based stereology (3D) is more reliable and accurate than 2D image analysis in quantitative analysis.(H. J. Gundersen, 1986; Schmitz & Hof, 2005; Selemon & Rajkowska, 2002) Several theoretical papers have discussed strength and weakness of model-based (2D) versus design-based (3D) in estimating total number of cells, and merits of design-based stereology far outweigh other 2D techniques in quantitative analysis.(Benes & Lange, 2001; Guillery & Herrup, 1997; H. J. Gundersen, 1978; von Bartheld, 2001; West, Slomianka, & Gundersen, 1991) Design-based stereology provides accurate method to quantify morphological and anatomical features in neuroscience. Regardless of theoretical considerations, 2D quantitative analyses remaining and have been commonly used to compare morphological and anatomical features between groups in animal models.(Anastasia, et al., 2009; Faherty, et al., 2005; Lebedev, Droblenkov, & Shabanov, 2008; Rinne, et al., 2008) However, there is lack of research to investigate the accuracy of 2D quantitative analysis to estimate the difference of morphological and anatomical features using identical groups. The purpose of this study is to compare 2D analysis with design-based stereology in quantifying differences of morphological and anatomical features between groups in three different rodent models.

Methods

Brain tissue samples of three different rodent models were analyzed; chronic MPTP/probenecid PD (MPD) mouse model, alcohol preferring (AP) rat model, and the enriched environment (EE) rat model. Chronic MPD mouse model was used as a severe model and two rat models were used as modest models. The severe model had relatively large difference of neuronal number between control and experimental groups as compared to the modest models. With the severe and modest models, the accuracy of estimation and the sensitivity for detecting differences were tested in brain samples of three different breeds including C57BL/6 mice, Wistar rats, and Sprague-Dawley rats. The tissue samples were embedded using the MultiBrain technologyTM, sectioned coronally and immunostained with tyrosine hydroxylase (TH) (NeuroScience Associates, Knoxville, TN).

Animals and tissue samples

Ten male C57BL/6 mouse brains were used for chronic MPD mouse model in this study. Five brains were obtained from chronic MPD mice and others from controls. To prepare the chronic MPD with moderate neurodegeneration, five mice were injected with a total of 10 doses of MPTP hydrochloride in combination with an adjuvant, probenecid on a 5-week schedule with an interval of 3.5 days between consecutive doses.(S. O. Ahmad, et al., 2009; Lau, Trobough, Crampton, & Wilson, 1990) For quantitative analyses, mice were anesthetized and transcardially perfused.(S. O. Ahmad, et al., 2009) The brain samples were embedded with the MultiBrainTM technology, sectioned coronally at 50 μ m and immunostained with tyrosine hydroxylase (TH) (NeuroScience Associates, Knoxville, TN). This method of preparation yielded minimal shrinkage (slices were cut at 50 μ m and reduced

to 30.87 μ m) and no evidence of tissue distortion.(S. O. Ahmad, et al., 2009)

Sixteen brains of adult female Wistar rats were analyzed for alcohol-preferring (AP) rat model in this study. These brains were obtained from eight alcohol-preferring (AP) and eight alcohol–nonpreferring (ANP) rats (Indiana University School of Medicine). The rats were not exposed to alcohol.(S.O. Ahmad, Park, Penick, & Manzardo, 2009)

Sixteen Sprague-Dawley rats (8 males, 8 females) were used for EE rat model. These 16 brains were obtained from 8 rats exposed to EE and 8 rats to standard environment (SE) for about 2 months.(Almli, et al., 2008) In EE, toys (objects of wood, metal, plastic, leather, and cardboard) were available at all times in the cage and items were alternated every three days for novelty. There were meshed wire ladders for climbing and plastic (PVC) pipes for tunneling and nesting. SE had no toys and were used in a transparent plastic laboratory cage lined with wood shavings.(Almli, et al., 2008)

For quantitative analyses, tissue preparation process was same in both AP rat model and EE rat model.(S.O. Ahmad, et al., 2009; Almli, et al., 2008) All rats were anesthetized and then perfused through the left ventricle.(Almli, et al., 2008) Brains were then post fixed and sectioned coronally at 60 μ m by NeuroScience Associates (Knoxville, TN) using MultiBrain[™] Technology. Free-floating sections were stained for tyrosine hydroxylase (TH). Samples were mounted on gelatinized (subbed) glass slides for viewing.(Almli, et al., 2008)

Apparatus

The *Stereologer* software package (Stereology Resource Center, Baltimore INC, Baltimore, MD) was used with a Nikon Eclipse 80i microscope, connected with a Sony 3CCD Color Digital Video Camera, which operated an Advanced Scientific Instrumentation

MS-2000 motorized Stage input into a Dell Precision 650 Server and a high resolution plasma monitor. The microscope has 4X, 20X, and 100X lenses. For 2D image analysis, the *Stereologer* software was used to capture 2D images of sample sections and then the NIH *ImageJ* software (National Institutes of Health, Bethesda, MD) was used to analyze 2D images of brain samples in neuronal number, neuronal volume, and region volume. For design-based stereology, the *Stereologer* software package was used in estimation of total neuronal number, neuronal volume, and region volume.

Data collection

The number of TH-positive neurons was quantified by design-based stereology, model-based stereology, the fractionator method and densitometry in the SNpc of chronic MPD mouse model, EE rat model and in the VTA of AP rat model. The volume of TH-positive neurons was quantified by design-based stereology, model-based stereology, and profile measure in the SNpc of chronic MPD mouse model, EE rat model and in the VTA of AP rat model. The region volume of the NAc in AP rat model was measured by design-based stereology, model-based stereology, and profile measure. The areas of interest located in the right hemisphere were analyzed in samples from animals in each model. Each analysis used the same sample sections in each model. For TH-positive neuronal number and volume, every 8th section containing the SNpc (Rat: Bregma -4.80 to -6.30mm, Mouse: Bregma -2.54 to -3.88mm) and the VTA (Rat: Bregma -5.20 to -6.30mm) was selected from a random initial sort. For regional volume, every 4th section containing the NAc (Bregma 2.70 to 0.70mm) was selected from a random initial sort.

2D analyses were used to quantify the number of TH-positive neurons in the area of interest. Images of each section were captured with 20X lens by the CCD camera connected to the microscope. All TH-positive neurons in the area of interest were counted on each

section using an image analysis program (NIH imageJ). Once sample sections of an animal were counted, the fractionator method estimated the total number of DA neurons by multiplying the total number of counted neurons by 8 to correct for uncounted sections. Model-based stereology applied the Abercrombie correction to correct for split neurons. (Abercrombie & Johnson, 1946) $N_t = N_a(T/D+T)$, where N_t = total number of neurons, N_a = total number of neurons from the fractionators method, D = average diameter of neurons, and T = section thickness. The section thickness (T) was 50 μ m in the chronic MPTP mice and 60 μ m in AP and EE rats. In model-based stereology, the 3D sphere diameter (D) of neurons was calculated for neuronal size from the average neuronal diameter (d) by the formula $D=4d/\pi$. (Rinne, et al., 2008; Weibel, 1979) The diameter (d) of neurons was estimated from the formula $d = \sqrt{4A/\pi}$. (Rinne, et al., 2008) The profile areas of neurons (A) were measured by imageJ software. In each section, areas of 10 neurons were measured to calculate the mean diameter of neuronal bodies. In addition to model-based stereology and the fractionator method, optical density was used to quantify the number of TH-positive neurons using ImageJ software. For each section image, the area of interest was outlined precisely according to a stereotaxic atlas. (Paxinos & Watson, 2007) Then optical density of the area of interest was assessed in each sample section using imageJ. The optical density of the interest area in an animal was the sum of optical densities in all sample sections.

2D analyses were used to quantify the volume of TH-positive neurons in the area of interest. The volume of neurons (V) was estimated from the formula $V = (4\pi/3)r^3$. The radius of neurons (r) was estimated by model-based stereology and profile measure. In model-based stereology, the 3D sphere radius (r) of neurons was estimated by dividing the 3D sphere diameter (D) of neurons by 2. In profile measure, the radius of neurons was measured by ImageJ. The largest diameter of the neuron was measured and then divided by 2. In both

analyses, neuronal volume was estimated from the average of ten neurons in each section.

The Cavalieri-area method (2D) was used to estimate the regional volume of the right NAc. Images of each section were captured with 4X lenses by the CCD camera connected to the microscope. The volume of the NAc was estimated by multiplying the sum of the NAc area in sample sections by the spacing distance (SD). The SD was calculated by multiplying the section interval (4: every 4th) by section thickness. The area of the NAc in each section was measured using ImageJ software. The two estimations of the NAc volume were generated with section pre stained cut thickness (60 μm) and actual section thickness. Actual section thickness was measured by linear encoder with 100X lens.

Design-based stereology estimated neuronal number, neuronal volume and regional volume. In design-based stereology (3D), the optical disector method was used to estimate the total number of TH-positive neurons in the area of interest. The counting process was as follows: First, each section was checked at low magnification (4X) and the area of interest was precisely outlined using stereotaxic atlases of mouse and rat brains. (Franklin & Paxinos, 2008; Paxinos & Watson, 2007) After outlining, the software applied systematic random grids to select counting frames on the area of interest. In each counting frame, TH-positive neurons were counted at high magnification with a 100X/1.4 aperture oil immersion lens (yielding 3600X) by the optical disector principle. The guard height was 3 μm and the thickness of each section was determined by the mean thickness of count frames measured by a 100X/1.4 aperture oil immersion lens. The nucleator method with number-weighted principle was used to estimate the neuronal volume of TH-positive neurons of the SNpc and the VTA on the right hemisphere. Just after counting the neuron in each counting frame, the volume of each counted neuron was measured with a 100X/1.4 aperture oil immersion lens. The Cavalieri-point counting method was used to estimate the total volume of the NAc. In each section, the

NAC was outlined with low magnification (4X) measured against a stereotaxic atlas.(Paxinos & Watson, 2007) After outlining, random grids were applied on the NAC by the software. Grids only within the NAC were manually selected. The thickness of each section was measured with a 100X/1.4 aperture oil immersion lens.

Data analysis

All data are presented as the mean \pm S.E.M. in actual estimation and percent (Table 1) (Table 2) (Table 3). All data were normalized in a percentage with respect to the mean of the control group in each model. Control groups were sedentary control (SC) in MPD mouse model, standard housing environment (SE) in EE rat model, and alcohol non-preferring (ANP) in AP rat model. Student's *t*-test (two-tailed) was used to compare quantitative data of each analysis between control and experimental groups in each model. Student's *t*-test (two-tailed) was also used to compare quantitative data between design-based stereology and 2D analyses. Differences between groups were considered significant at $p<0.05$.

Results

In TH⁺ neuronal number of chronic MPD model, all quantitative analyses found significant differences between SC and MPD in both actual number and normalized value (Figure 4). In actual number, model-based stereology demonstrated a significant difference in SC from design-based stereology. In normalized data, 2D analyses presented a significantly larger percentage of TH-positive neurons than design-based stereology in MPD. In EE rat model, design-based stereology found a significant difference of neuronal number between SE and EE but 2D analyses did not (Figure 5). In actual number estimation, model-based stereology demonstrated a smaller number than design-based stereology while the fractionator method generated a larger number. Neuronal numbers of EE, estimated by model-based stereology, and neuronal number of SE, estimated by the fractionator method, were significantly different from neuronal number estimated by design-based stereology. In normalized data, design-based stereology found a significant difference between SE and EE while 2D analyses did not. Normalized data of EE in design-based stereology was significantly different from all 2D analyses: model-based stereology, the fractionator method, and densitometry. In AP rat model, design-based stereology demonstrated a significant difference of neuronal number between ANP and AP but all 2D analyses did not in both actual number estimation and normalized value (Figure 6). In actual number, model-based stereology and the fractionator method estimated significantly a smaller number than design-based stereology in both groups. In normalized data, there is no significant difference between design-based stereology and 2D analyses: model-based stereology, the fractionator method, and densitometry.

In TH⁺ neuronal volume of chronic MPD model, there was no significant difference between SC and MPD (Figure 7). In actual volume estimation, model-based stereology and

profile measure demonstrated significantly larger volume than design-based stereology. In normalized data, the estimation of MPD by profile measure was significantly smaller than the estimation of MPD by design-based stereology. In EE rat model, there was no significant difference between SE and EE (Figure 8). In actual volume estimation, both model-based and profile measure demonstrated significantly larger volume than design-based stereology in both SE and EE. In normalized data, there was no significant difference between design-based stereology and 2D analyses: model-based stereology and profile measure. In AP rat model, there was no significant difference between ANP and AP (Figure 9). In actual volume estimation, both model-based and profile measure demonstrated significantly larger volume than design-based stereology in both ANP and AP. In normalized data, there was no significant difference between design-based stereology and 2D analyses: model-based stereology and profile measure.

In the regional volume of the NAc, all analyses found a significant difference between ANP and AP (Figure 10). In actual volume, model-based stereology generated two estimations. The estimation with section pre-stained thickness demonstrated significantly larger volume in both ANP and AP but the estimation with actual thickness measured by linear encoder during the 3D process were not significantly different from estimation of design-based stereology. In normalized value, there was no difference between design-based stereology and 2D analyses.

Table 1. Quantification of TH+ neuronal number

Models	Group	Design-based stereology	Model-based stereology	Fractionator method	Densitometry
MPD	Control (SC)	5272.28±489.38 (100±9.45)	3034.50±257.06 ⁺ (100±8.47)	4046.00±342.74 (100±8.47)	100±6.45
	Experimental (MPD)	2393.86±242.46* (45.40±4.59*)	2097.60±184.20* (65.31±3.87* ⁺)	2796.80±245.60* (65.31±3.87* ⁺)	76.03±3.90* ⁺
EE	Control (SE)	4144.84±244.31 (100±5.89)	3855.40±160.43 (100±4.16)	5210.00±246.80 ⁺ (100±4.16)	100±5.56
	Experimental (EE)	4853.59±198.15* (117.09±4.78*)	3974.54±111.79 ⁺ (103.09±2.89 ⁺)	5371.00±151.07 (103.09±2.89 ⁺)	95.62±3.23 ⁺
AP	Control (ANP)	8683.95±488.63 (100±5.62)	4037.88±378.66 ⁺ (100±9.37)	5244.00±491.77 ⁺ (100±9.37)	100±5.50
	Experimental (AP)	10457.25±494.16* (120.42±5.69*)	4375.80±490.58 ⁺ (108.36±12.14)	5610.11±628.94 ⁺ (106±11.99)	110±5.12

Indicated as percentage of control (mean ± SEM); *p<0.05 vs control, ⁺p<0.05 vs design-based stereology

Table 2. Quantification of TH+ neuronal volume (μ³)

Models	Group	Design-based stereology	Model-based stereology	Profile measure
MPD	Control (SC)	1129±76.33 (100±6.76)	3995.04±219.45 ⁺ (100±5.49)	3888.91±123.05 ⁺ (100±3.16)
	Experimental (MPD)	1157±33.93 (102±3.00)	4012.35±162.70 ⁺ (100.43±4.07)	3496.31±148.87 ⁺ (89.90±3.82 ⁺)
EE	Control (SE)	1381.36±54.90 (100±3.97)	5011.96±184.82 ⁺ (100±3.68)	3645.55±118.89 ⁺ (100±3.26)
	Experimental (EE)	1403.73±109.51 (101.61±7.92)	5074±314.40 ⁺ (101.25±6.27)	3323.05±191.26 ⁺ (91.15±5.24)
AP	Control (ANP)	546.58±37.01 (100±6.77)	3028.71±169.05 ⁺ (100±5.58)	1487.31±219.73 ⁺ (100±7.37)
	Experimental (AP)	558.23±40.05 (102.13±7.32)	2758.47±162.44 ⁺ (91.07±5.36)	1734.33±67.23 ⁺ (116±4.52)

Indicated as percentage of control (mean ± SEM); *p<0.05 vs control, ⁺p<0.05 vs design-based stereology

Table 3. Quantification of NAc volume (mm³)

Models	Group	Design-based stereology	Model-based stereology I	Model-based stereology II	Profile measure (mm ²)
AP	Control (ANP)	2.13±0.10 (100±6.10)	2.94±0.05 ⁺ (100±1.71)	1.96±0.03 (100±1.71)	12.27±0.21 (100±1.71)
	Experimental (AP)	1.76±0.11* (82.89±5.22*)	2.46±0.10* ⁺ (83.52±3.63*)	1.64±0.07* (83.52±3.63*)	10.25±0.44 (83.52±3.63*)

Indicated as percentage of control (mean ± SEM); *p<0.05 vs control, ⁺p<0.05 vs design-based stereology

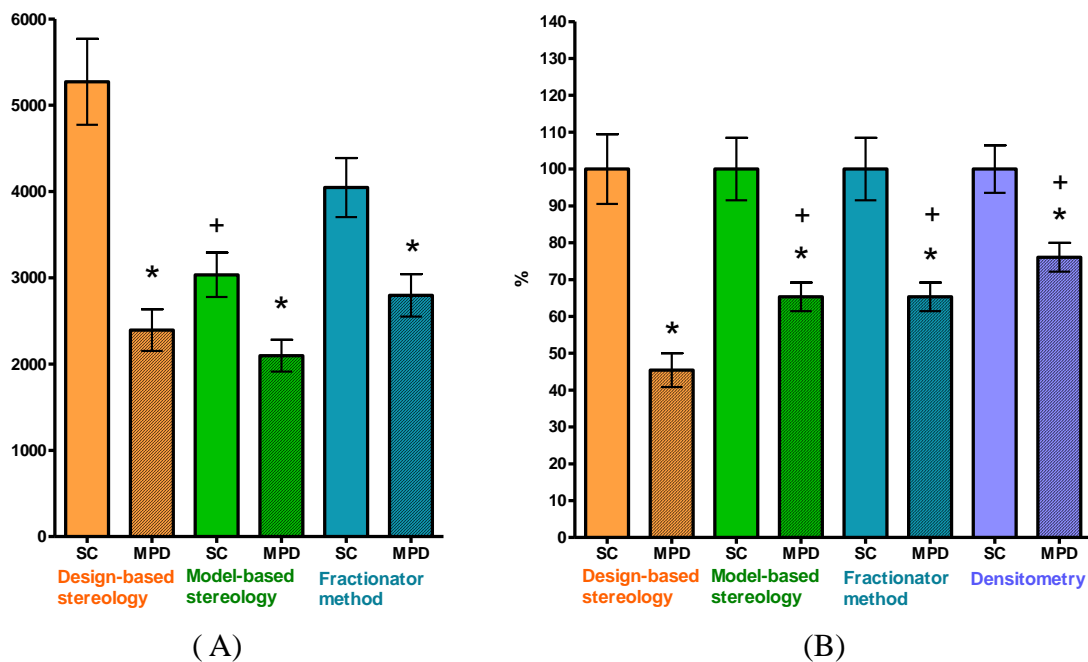


Figure 4. Quantification of TH⁺ neuronal number in the SNpc of MPD mouse model (mean±SEM). SC, sedentary control; MPD, chronic MPTP/probenecid PD. **(A)** Actual number of neurons was estimated by three quantification methods: design-based stereology, model-based stereology, and the fractionator method. All three found significant DA neuronal loss in the SNpc of MPD. Both model-based stereology and the fractionator method estimate fewer neuronal number than design-based stereology. Only neuronal number of SC in model-based stereology was significantly different from the number of SC in design-based stereology. * $p < 0.05$ vs SC. + $p < 0.05$ vs design-based stereology. **(B)** Neuronal number was normalized in a percentage as respect the mean of the SC. All four found significant differences of neuronal number of DA neurons in the SNpc. However, three 2D methods, model-based stereology, the fractionator method, and densitometry, demonstrated fewer neuronal loss of DA neurons than design-based stereology. * $p < 0.05$ vs SC. + $p < 0.05$ vs design-based stereology.

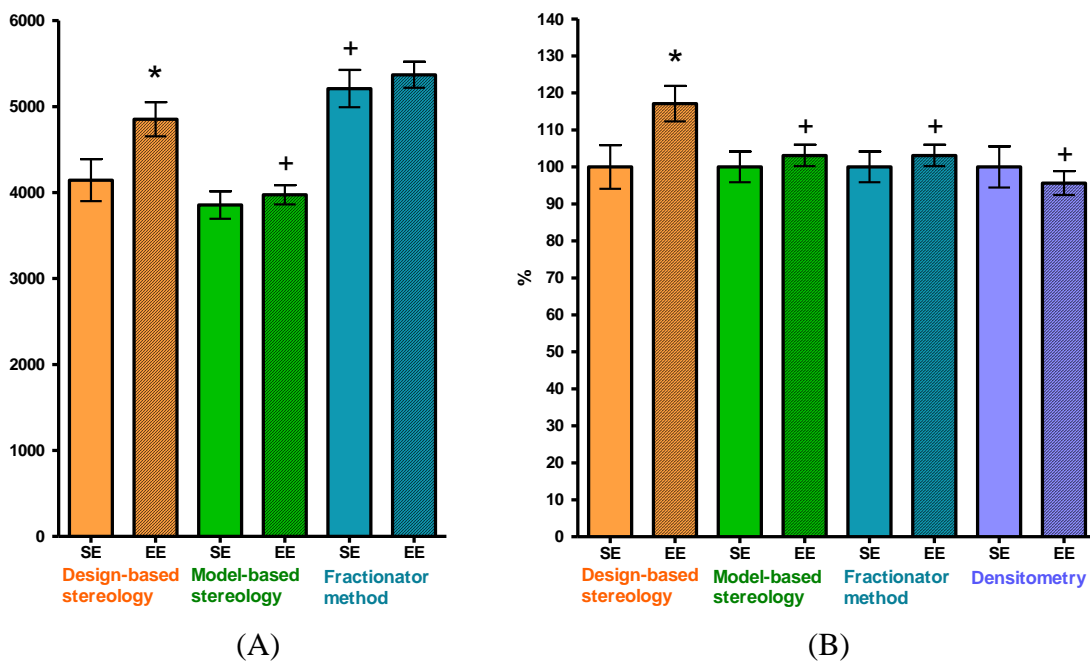


Figure 5. Quantification of TH+ neuronal number in the SNpc of EE rat model (mean±SEM). SE, standard environment; EE, enriched environment. **(A)** Actual number of neurons was estimated by three quantification methods: design-based stereology, model-based stereology, and the fractionator method. Only design-based stereology found a significant difference of DA neuronal number between SE and EE. 2D analyses, including model-based stereology, and the fractionator method, did not find significant differences between SE and EE. DA neuronal number in EE of model-based stereology and SE of the fractionator method were significantly different from DA neuronal number in design-based stereology. * $p < 0.05$ vs SE. + $p < 0.05$ vs design-based stereology **(B)** Neuronal number was normalized in a percentage as respect the mean of the SE. Only design-based stereology found a significant difference of DA neuronal number between SE and EE. All 2D analyses, including model-based stereology, the fractionator method, and densitometry, were significantly different from design-based stereology in EE. * $p < 0.05$ vs SE. + $p < 0.05$ vs design-based stereology.

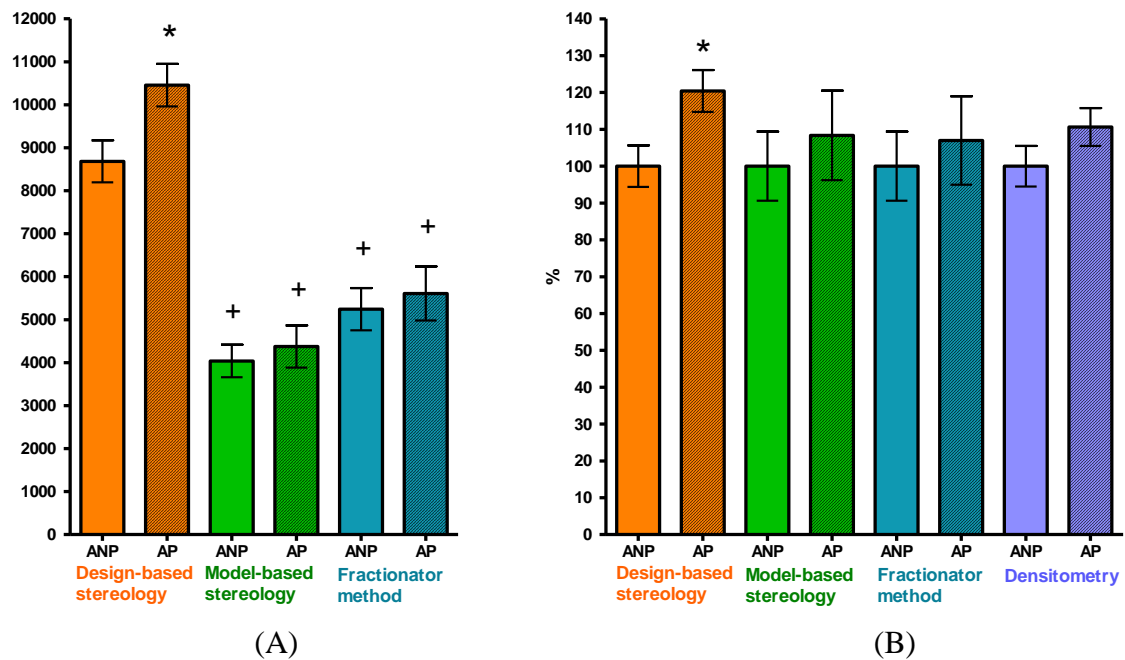


Figure 6. Quantification of TH+ neuronal number in the VTA of AP rat model (mean±SEM). ANP, alcohol non-preferring; AP, alcohol preferring. **(A)** Actual number of DA neurons was estimated by design-based stereology, model-based stereology, and the fractionator method. Only design-based stereology found a significant difference of DA neuronal number between ANP and AP. 2D analyses, including model-based, and the fractionator method, did not find a significant difference between ANP and AP. All 2D analyses demonstrated less neuronal number than design-based stereology. All neuronal numbers estimated by 2D analyses were significantly different from neuronal numbers estimated by design-based stereology. * $p < 0.05$ vs ANP, + $p < 0.05$ vs design-based stereology. **(B)** Neuronal number was normalized in a percentage as respect the mean of ANP. Only design-based stereology found a significant difference in DA neuronal number between ANP and AP. There is no significant difference between design-based stereology and 2D analyses. * $p < 0.05$ vs ANP, + $p < 0.05$ vs design-based stereology.

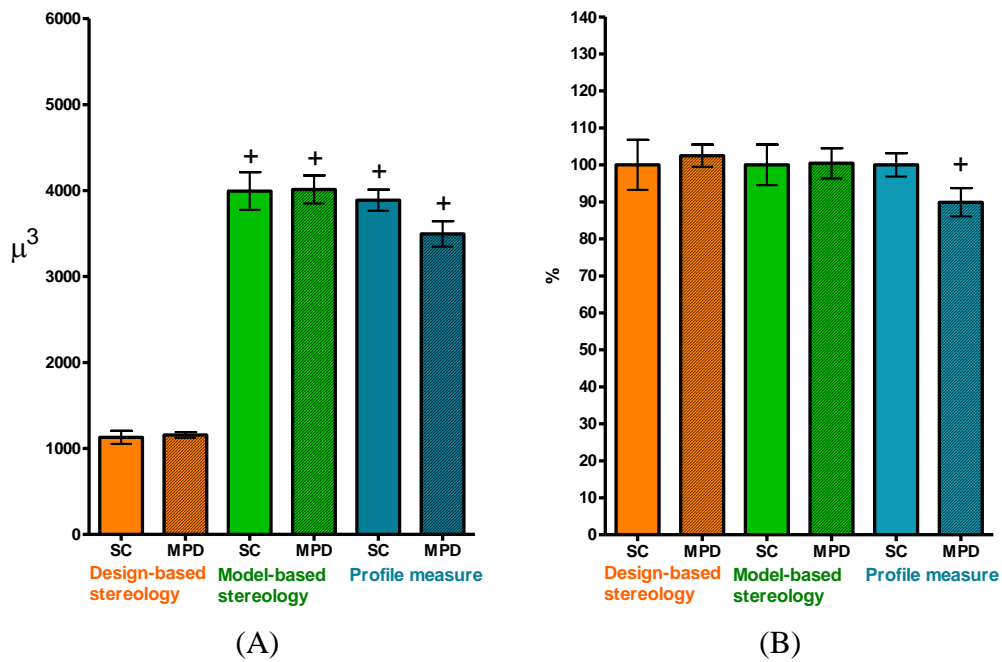


Figure 7. Quantification of TH+ neuronal volume in the SNpc of MPD mouse model (mean \pm SEM). SC, sedentary control; MPD, chronic MPTP/probenecid PD. (A) Actual neuronal volume was estimated by design-based stereology, model-based stereology, and profile measure. All three analyses did not find a significant difference of neuronal volume between SC and MPD. 2D analyses, including model-based stereology and profile measure, estimated significantly different from design-based stereology in both SC and MPD. * $p < 0.05$ vs SC, + $p < 0.05$ vs design-based stereology. (B) Neuronal volume was normalized in a percentage as respect the mean of the SC. All three analyses did not find significant differences of neuronal volume between SC and MPD. MPD of profile measure was significantly different from MPD of design-based stereology. * $p < 0.05$ vs SC, + $p < 0.05$ vs design-based stereology.

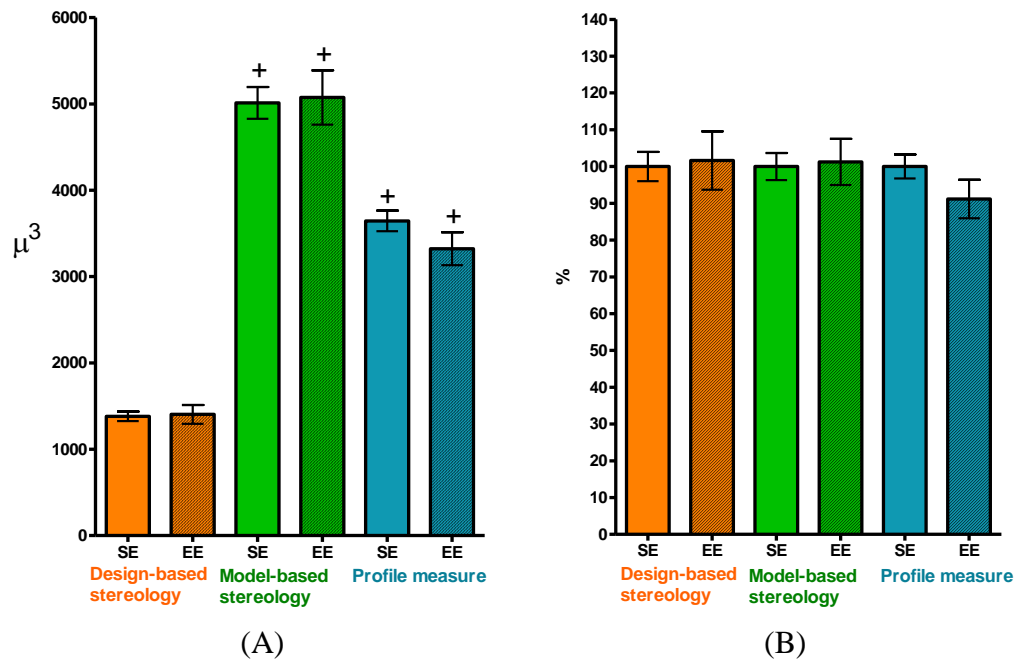


Figure 8. Quantification of TH+ neuronal volume in the SNpc of EE rat model (mean \pm SEM). SE, standard environment; EE, enriched environment. **(A)** Actual neuronal volume was estimated by design-based stereology, model-based stereology, and profile measure. All three methods did not find significant differences between SE and EE. 2D analyses, including model-based stereology and profile measure, demonstrated less neuronal volume significantly than design-based stereology in both SE and EE. * $p < 0.05$ vs SE, + $p < 0.05$ vs design-based stereology. **(B)** Neuronal volume was normalized in a percentage as respect the mean of the SE. All three methods did not find a significant difference between SE and EE. There is no significant difference between design-based stereology and 2D analyses. * $p < 0.05$ vs SE, + $p < 0.05$ vs design-based stereology.

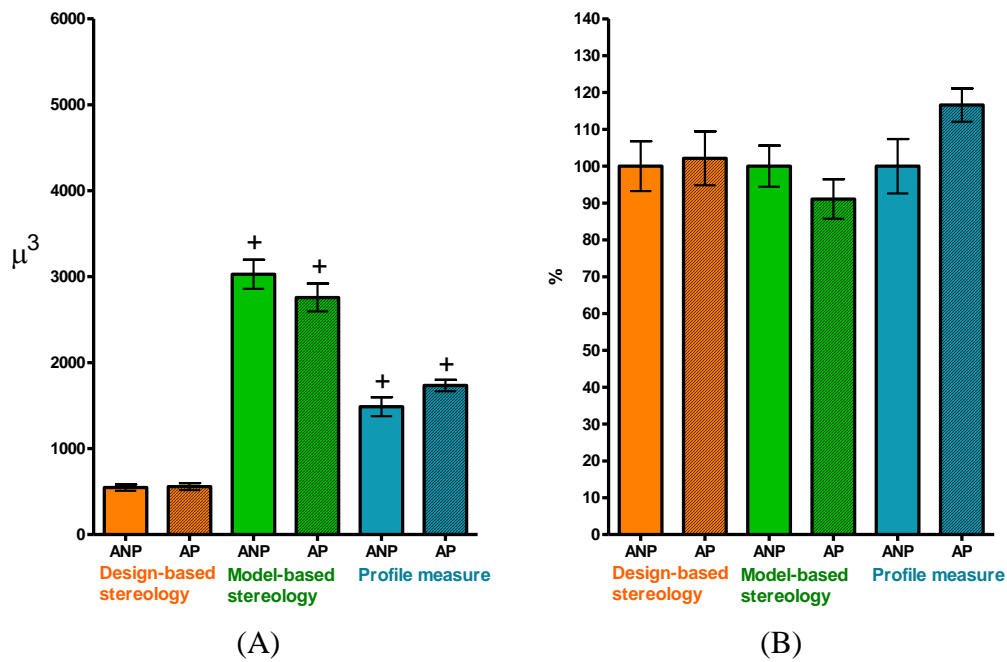


Figure 9. Quantification of TH+ neuronal volume in the VTA of AP rat model (mean \pm SEM). ANP, alcohol non-preferring; AP, alcohol preferring. **(A)** Actual neuronal volume was estimated by design-based stereology, model-based stereology, and profile measure. All three methods did not find a significant difference between SE and EE. 2D analyses, including model-based stereology and profile measure, demonstrated larger neuronal volume significantly than design-based stereology in both ANP and AP. * $p < 0.05$ vs ANP, + $p < 0.05$ vs design-based stereology. **(B)** Neuronal volume was normalized in a percentage as respect the mean of the SE. All three methods did not find a significant difference between SE and EE. There is no difference between design-based stereology and 2D analyses. * $p < 0.05$ vs ANP, + $p < 0.05$ vs design-based stereology.

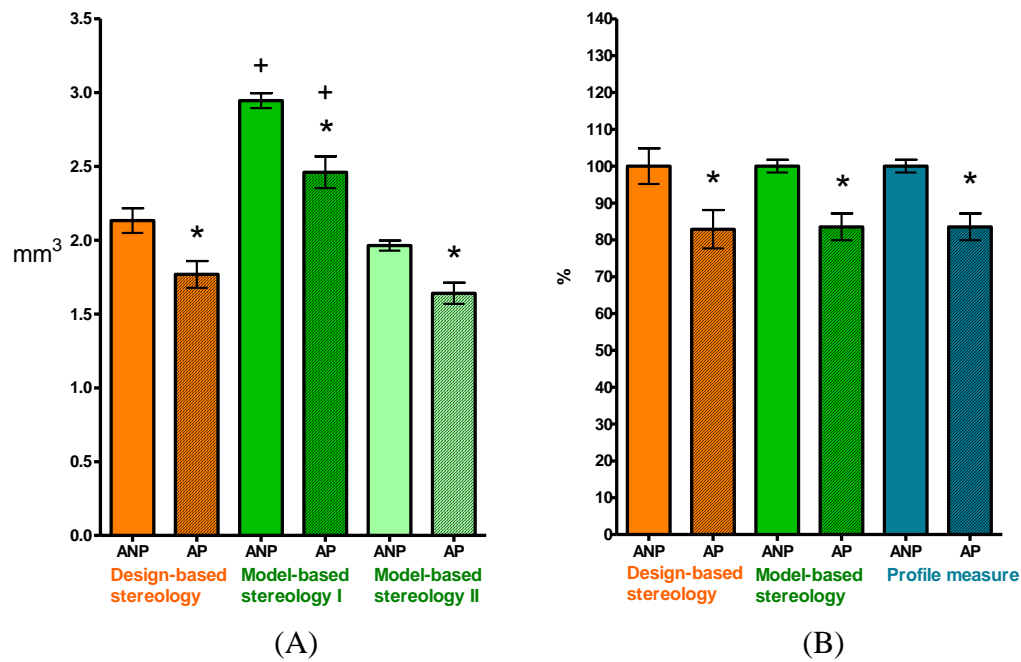


Figure 10. Quantification of NAc volume in the VTA of AP rat model (mean \pm SEM). NAc, nucleus accumbens; ANP, alcohol non-preferring; AP, alcohol preferring. **(A)** Regional volume of the NAc was estimated by design-based stereology and model-based stereology. Model-based stereology I used pre stained cut thickness (60 μ) and model-based stereology II used actual section thickness (40 μ) for estimation. All three estimation demonstrated significant differences between ANP and AP. Model-based stereology I showed significantly larger regional volume of the NAc than design-based stereology. *p<0.05 vs ANP, +p<0.05 vs design-based stereology **(B)** The volume of the NAc was normalized in a percentage as respect the mean of the ANP. All three methods found significant differences between ANP and AP. There is no significant difference between design-based stereology and 2D analyses. *p<0.05 vs ANP, +p<0.05 vs design-based stereology

Discussion

Design-based stereology has been used as the gold standard for quantifying morphological and anatomical features in light microscopy.(Saper, 1996; West & Gundersen, 1990) For the estimation of neuronal number, design-based stereology was not significantly different from the estimation of 3D reconstruction of serial sections in DA neurons of SNpc in the C57B/6J mouse.(Baquet, et al., 2009) 3D reconstruction of serial sections provides an accurate determination of neuronal number by counting all neurons in the interest area at hand.(Coggeshall & Lekan, 1996; Guillery & Herrup, 1997) In design-based stereology, variations in cell size, cell shape, and section thickness do not influence the estimation of total neuronal number as it does in estimations using 2D analyses.(Selemon & Rajkowska, 2002) These theoretical disadvantages of 2D analyses have led to the development of design-based stereology but 2D analyses are still commonly used in comparison of morphological and anatomical features.(Schmitz & Hof, 2005; Sterio, 1984)

The results of this study in quantification of neuronal number suggest that 2D analyses may be adequate for rough screening but are inaccurate and unreliable for the estimation of total neuronal number. Model-based stereology significantly underestimated actual neuronal number compared to design-based stereology in all three models. The fractionator method underestimated neuronal number in both chronic MPD mouse model and AP rat model but overestimated in EE rat model. Comparing the difference between control and experimental groups, 2D analyses found a significant difference in chronic MPD mouse model as a severe model but not in modest models such as EE rat model and AP rat model. However, design-based stereology found significant differences in all three models. Thus, 2D analyses are not accurate or reliable in estimating total neuronal number but it may detect relatively large differences between groups.

In quantification of neuronal volume, the result of the present study implies that 2D analyses may be reliable in normalized data but inaccurate in actual volume. Model-based stereology and profile measurement demonstrated significant overestimation of neuronal volume in all three models. In normalized data of neuronal number, 2D analyses demonstrated no significant difference from design-based stereology in control and experimental groups except for the estimation of profile measurement in MPD. Even though 2D analyses produced inaccurate neuronal volume estimation, normalized data from model-based stereology seemed to be reliable for a rough comparison or for large effects.

The results of this study in quantification of regional volume suggest that 2D analyses are accurate for estimating regional volume. Model-based stereology with actual section thickness demonstrated no significant difference from design-based stereology in the estimation of regional volume but significantly overestimated the regional volume with section pre stained thickness. Design-based and model-based stereology found a significant difference between ANP and AP regardless of overestimation with the use of section pre stained thickness. In normalized value, all analyses found a significant difference in the regional volume of the NAc between ANP and AP and there is no significant difference in normalized value between design-based stereology and 2D analyses such as model-based stereology and profile measure. Thus, model-based stereology accurately estimates regional volume using real section thickness rather than section pre stained thickness and profile measure generated comparable value in normalized data. The overall results of this study suggest that 2D quantitative analyses may be adequate to find relatively large differences in neuronal number and volume but are not accurate and reliable for estimation of total number and neuronal volume in rodent mesolimbic system.

There are several possible reasons for the difference of estimations in total neuronal

number between design-based stereology and 2D analyses. Model-based stereology and the fractionator method estimate total neuronal number from the number of neurons counted in 2D profile images. Many theoretical papers have argued that the major error in the use of profile counting is due to split-cells because split neurons have a high probability to be counted more than one time. (Abercrombie & Johnson, 1946; Baquet, et al., 2009; Selemon & Rajkowska, 2002; West & Gundersen, 1990) This bias from split-cells causes overestimation of total neuronal number using the fractionator method. The fractionator method overestimates total number of DA neurons by approximately 70% in samples with 10 μ sections as compared to design-based stereology in the SNpc of the C57BL/6J mouse. (Baquet, et al., 2009) Overestimation due to split-cells depends on section thickness. The fractionator method overestimates more in thin sections than in thick sections because thin sections generate more split-cells. (Cooper & Sofroniew, 1996; Meredith, Dudenhoeffer, & Jackson, 1999) However, the present study demonstrated the fractionator method did not always overestimate neuronal number because cross-sectional images of a sample section may not represent whole neurons of sample tissue in thick section. In the present study, thickness of sample tissue was 50 μ (mouse) or 60 μ (rat) and the diameter of neurons were about 10 μ . The thickness of section was 5 or 6 times greater than the diameter of neuron. Thick sections of sample tissue may cause underestimation of neuronal number and attenuate overestimation due to split-cells because some neurons may overlap and become unfocused in the Z-axis perspective. Neuronal size affects these biases. Smaller neurons have less probability to be split and may be more unfocused neurons in thick section, which may explain the reason why 2D analyses demonstrated significant underestimation of neuronal number in AP rat model. In this model, neuronal volume was about two times smaller than neuronal volume of the other studied models.

The Abercrombie correction factor did not adjust correctly for neuronal number estimation. Model-based stereology applies the Abercrombie correction factor to compensate for overestimation due to split-cells from the fractionator method.(Abercrombie & Johnson, 1946; Guillery & Herrup, 1997) The present study demonstrated model-based stereology with Abercrombie correction was not reliable in the estimation of neuronal number. Larger sampling intervals may cause a loss of precision in estimation of 2D neuronal counting method with the Abercrombie correction. Sample intervals larger than 150 μ have shown to cause inaccuracy in estimating numbers of TH-positive neurons in the SNpc of the C57BL/6J mouse.(Baquet, et al., 2009) Appropriate sampling intervals for 2D analyses should be less than 100 μ m. In the present study, the sampling interval was 400 μ m for chronic MPD mouse model and 480 μ m for both EE rat model and AP rat model. This larger sampling interval may underestimate total neuronal number in the present study because of insufficient sample sections. The Abercrombie correction factor compensates for overestimation due to split-cells but not for underestimation due to thick sections coupled with large sample intervals. Thus, the Abercrombie correction cannot compensate correctly for biases causing underestimation of neuronal number in the fractionator method and model-based stereology. In addition, the Abercrombie correction demonstrated an unreliable adjustment of overestimation.(Baquet, et al., 2009; Meredith, et al., 1999) These biases affect not only estimation of total number but also normalized data for comparison in the ratio of the experimental group to the control group.

Normalized data of 2D analyses demonstrated inaccurate estimations of differences between comparison groups in neuronal number. Model-based stereology and the fractionator method estimated about 35% neuronal loss while design-based stereology estimated 55% loss in chronic MPD mice. Densitometry also underestimated neuronal loss. Densitometry

presented more bias in the estimation of neuronal number by percentage because densitometry quantified not only TH-positive neurons, but also other structures stained such as dendrites. Normalization by percentage did not compensate for biases due to section thickness and section interval in model-based stereology and the fractionator method. Both model-based stereology and the fractionator method presented the same estimations in normalized value because the Abercrombie correction factor does not affect the ratio between comparison populations if neuronal size was not changed. (Guillery & Herrup, 1997; Selemon & Rajkowska, 2002) Neuronal size was not significantly different between control and experimental groups in each model. The correction factor was almost identical (0.75) in both groups. Thus, the normalized data of neuronal number in the fractionator method and model-based stereology was not changed after applying the Abercrombie correction factor. Normalization did not compensate for the bias of neuronal counting in 2D analyses such as model-based stereology and the fractionator method. Previous studies demonstrated similar differences in estimation between design-based stereology and 2D analyses on chronic MPD mice model with the same breed and induced Parkinsonism protocol. Profile counting estimated about 30% neuronal loss but design-based stereology estimated about 50% neuronal loss. (Petroske, Meredith, Callen, Totterdell, & Lau, 2001; Schintu, et al., 2009) This difference may be caused not only by quantification methods but also other factors such as tissue preparation, spacing distance, and section thickness. The present study supports that different outcomes may be due to the inaccuracy of 2D analyses using same samples to reduce other biases and confounding variables for estimation. Thus, 2D analyses are inaccurate in both total number estimation and normalized value. The inaccuracy due to 2D count method affects sensitivity of 2D analyses in comparison to find differences between groups. Some researchers argued that model-based stereology can estimate accurately the ratio of experimental to control for comparison as long as neurons do not change in size,

shape, or orientation between groups.(Saper, 1996) The results of this study show that model-based stereology estimates are inaccurate for neuronal number even though there is no difference of neuronal volume between groups because for the profile ratio was not accurate and the Abercrombie correction did not compensate profile ratio. Neither profile counts nor profile ratios for comparison are accurate in neuronal number.(Coggeshall & Lekan, 1996) 2D analyses based on profile counts should be considered inaccurate. The results of present study show that model-based stereology and the fractionator method, which estimates total number of neurons from profile counts, are not accurate and reliable in total neuronal number and normalized data.

The present findings indicate that 2D analyses do not have enough sensitivity to detect relatively small differences in neuronal number. 2D analyses did not identify the relatively small differences in neuronal number of the SNpc. The severe model (MPD) demonstrated over than 50% difference between control and experimental groups while modest models (EE, AP) demonstrated less than 20% difference. 2D analyses, including model-based stereology, fractionator method, and densitometry, found a significant difference between control and experimental groups in chronic MPD model as a severe model but not in the modest models. Several studies quantified TH⁺ neurons in the SNpc to investigate the effect of EE in many rodent species such as C57B1/6 mice, SAMP8 mice, Wistar rats, and Sprague-Dawley rats.(Bezard, et al., 2003; Faherty, et al., 2005; Steiner, et al., 2006; Yuan, et al., 2009) In the intact rodent brain, most of 2D analyses demonstrated that there was no significant difference of neuronal number between control and EE groups in the SNpc but design-based stereology found significant differences.(Bezard, et al., 2003; Faherty, et al., 2005) Also, 2D analyses did not find a significant difference between ANP and AP rats that design-based stereology identified.(S.O. Ahmad, et al., 2009; Zhou, et al., 1995) The present

study demonstrates the low sensitivity of 2D analyses may produce different results relative to use of design-based stereology. However, 2D analyses generated a relatively accurate estimation in neuronal number and regional volume.

2D analyses overestimated the volume of individual neurons but generated comparable value to design-based stereology in normalized value. Both model-based stereology and profile measure demonstrated overestimation in neuronal volume. Design-based stereology and 2D analyses use the same equation to estimate neuronal volume, but use different methods to estimate the radius of the neuron. In design-based stereology, the nucleator method estimates the radius by averaging four radiuses obtained by applying two random lines on the center of the neuron. Model-based stereology estimates the radius by measuring the profile area of the neuron. Profile measure estimates the radius by measuring the largest diameter in the profile of the neuron. Relatively small overestimations of the radius generate relatively large overestimation of the volume because the radius is cubed in the formula for volume estimation, $V = (4\pi/3)r^3$. Because of overestimation in the radius of the neurons, the estimation of neuronal volume was significantly larger in 2D analyses than in design-based stereology. However, normalized values for comparison in 2D analyses were generally not significantly different from design-based stereology. The normalized data of model-based stereology were not significantly different from the estimations of design-based stereology in neuronal volume of experimental groups while the normalized of profile measure was significantly different in neuronal volume of MPD mice. Among 2D analyses, model-based stereology generated comparable value of neuronal volume in normalized data. However, it is not clear that model-based stereology has enough sensitivity to determine differences in neuronal volume because there was no significant difference of neuronal volume between control and experimental groups in all three models. Thus, 2D analyses

generated comparable value in normalized data of neuronal volume for comparison but not accurate estimation of neuronal volume.

2D analyses demonstrated accurate estimation of regional volume. With actual measured section thickness, model-based stereology estimated regional volume accurately but overestimated it with section pre stain cut thickness because sample tissue dehydrated after tissue preparation. Theoretically, the Cavalieri-point counting method was derived from the Cavalieri-area method, model-based stereology. Both design-based and model-based stereology estimated regional volume from profile area measure, so normalized values for comparison are not significantly different from each other. In the comparison of regional volume, model-based stereology and profile measure produced exactly the same result because the spacing distances were the same in ANP and AP rats. The volume of the NAc was estimated by multiplying the sum of the NAc area in sample sections (profile measure) by the spacing distance (Sd). $Sd = \text{section interval (4: every 4}^{\text{th}}) \times \text{section pre stained cut thickness (60 } \mu\text{m)}$. Sd was 240 μ in both ANP and AP groups. The ratio of ANP to AP in model-based stereology was same in profile measure, measuring area of the interest area on the cross-sectional profile. Thus, 2D analyses generated accurate value in the estimation of regional volume and in normalized data for comparison.

In summary, 2D analyses inaccurately estimated the total neuronal number and volume but accurately estimated regional volume. 2D analyses may be used for rough comparison of neuronal number and volume. Among 2D analyses, model-based stereology generated more similar estimations to design-based stereology than other 2D analyses such as densitometry and profile measure in neuronal number and volume. In regional volume, model-based stereology estimates accurately if actual thickness, as measured by line encoding, was used in calculation and profile measure is reliable and accurate in normalized

value. The results of the present study suggest that significant differences of neuronal number detected in previous studies using 2D analyses may be accepted but the estimation of a differences or actual value should not. The quantity of neuronal number is regarded as an indicator of severity of pathology for the effect size of interventions in degenerative diseases. Inaccurate estimation of differences between comparison groups may lead to incorrect assumptions on the severity of pathology or intervention effects. The results of this study also suggest future investigations should use design-based stereology for comparison of neuronal number in any model. For neuronal number, it seems appropriate to use model-based stereology for comparison of neuronal volume with normalized data but this present study could not test the estimation of difference and sensitivity because there was no significant difference of neuronal volume in each model. It is completely reasonable to use 2D analyses for comparison of regional volume in normalized value regardless of section thickness.

In conclusion, 2D analyses, including model-based stereology, and the fractionator method did not generate an accurate estimation of neuronal number because various factors distort accuracy of estimation such as sample interval, section thickness, neuronal size, and neuronal density. Despite inaccuracy, 2D analyses seem to be acceptable to be used for rough discriminations to find the difference of neuronal number. In neuronal volume, model-based stereology demonstrated comparable value in normalized data. In regional volume, model-based stereology can be used for estimating regional volume with real section thickness and profile measure can be used for comparison of regional volume. These results suggest new implication of previous studies in 2D analyses and that investigators should use design-based stereology rather than 2D analyses for comparison of morphological and anatomical features in brain. This study did not test sensitivity of 2D analyses and accuracy in a difference of neuronal volume, which could be the topic of a future study.

Conclusions

Design-based stereology (3D) is widely accepted as the gold standard of light microscopic morphometric analyses in the brain. Theoretical disadvantages of 2D analyses have led researchers to develop design-based stereology but many investigators still use 2D analyses in neuroscience because design-based stereology requires the purchase of expensive equipment and extensive training of the investigator. The present study compared 2D analysis versus design-based stereology to quantify morphological and anatomical features in three different rodent models.

The overall results of this study support the following: 1) 2D analyses (model-based stereology, fractionator method) inaccurately estimate total neuronal numbers compared to design-based stereology, 2) 2D analyses (model-based stereology, fractionator method, densitometry) could be used to determine relatively large differences of neuronal number, 3) 2D analyses (model-based stereology, profile measure) inaccurately overestimate neuronal volume, 4) 2D analyses (model-based stereology) generate comparable value in normalized data of neuronal volume to design-based stereology, and 5) 2D analyses (model-based stereology) estimate accurately regional volume as well as design-based stereology. 2D analyses generate inaccurate estimation of actual value of neuronal number and volume while 2D analyses are accurate in regional volume. Profile area measure is enough for comparison of regional volume. Among 2D analyses, model-based stereology has relatively less bias than other 2D analyses such as densitometry and profile measure in comparison of neuronal number and volume.

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Appendix A: Comprehensive Literature Review (I)

WHAT IS THE RELATIONSHIP OF ANATOMICAL STRUCTURES AND FUNCTIONS
OF THE SUBSTANTIA NIGRA AND THE VENTRAL TEGMENTAL AREA?

Ji-Hyuk Park

I. General overview of extra-pyramidal system

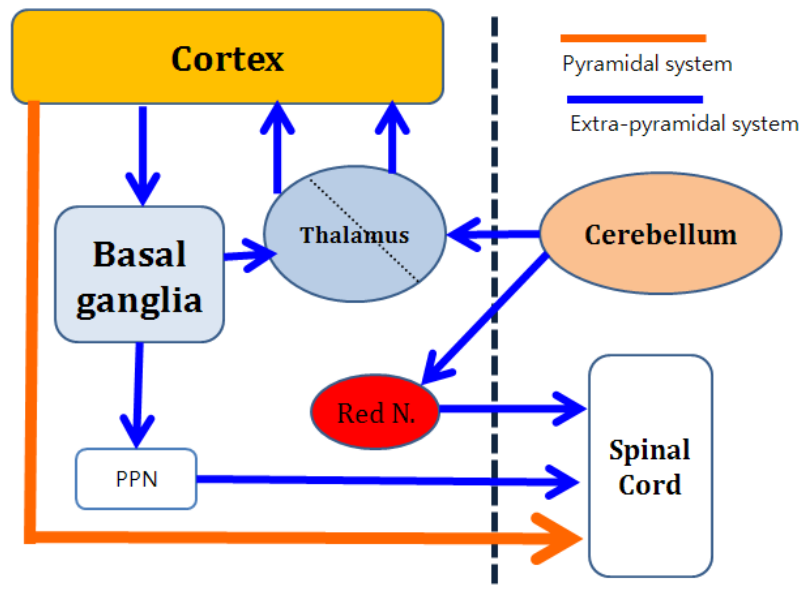


Figure 1. Pyramidal and extra-pyramidal system

The extra-pyramidal system is a neural system involved in regulating motor function. The British neurologist S. A. Kinnier Wilson first mentioned the “extra-pyramidal” motor system illustrating a disease with muscular rigidity, tremor, and weakness.(Wilson, 1928) The extra-pyramidal motor system was thought to contain coordination and postural control functions and to be independent of the pyramidal motor system. Thus, the motor deficits, characterized by involuntary movements, muscular rigidity, and immobility without paralysis, is called extra-pyramidal track syndrome. Wilson believed the basal ganglia(BG) were the key component of extra-pyramidal motor system.(Kandel, Schwartz, & Jessell, 2000; Squire, 2003b) Modern neuroscientists believe that pyramidal and extra-pyramidal system are not independent of each other, but interconnected and work in concert with each other to control of movement.(Kandel, et al., 2000) Nevertheless, the traditional phrase “extra-pyramidal”

system is still used to describe the BG and deficits of BG.(Squire, 2003b) Literally the extra-pyramidal motor system is a neural network involving all motor function except the pyramidal system which contains the corticospinal pathway.

The major structures of extra-pyramidal tract, such as the cerebellum and the BG, are involved in many brain functions. Both structures receive inputs from the cerebral cortex and then give feedback to the cortex via the thalamus, and have separate connections with the spinal cord.(Kandel, et al., 2000; Squire, 2003b) The cerebellum sends the output to the spinal cord through the red nucleus, whereas the basal ganglia sends information to the spinal cord via the pedunculopontine nucleus(PPN). The BG plays an important role in movement sequences and planning motor strategies. The cerebellum engages in assisting to compare cortex motor commands with proprioceptive input and generating coordinated movement.(Kandel, et al., 2000; Squire, 2003b) In addition to the motor function of these parts of the extra-pyramidal tract, recent studies uncovered that these major parts of the extra-pyramidal system can also affect various cognitive functions such as emotional expression, addictive behavior, and executive function.(Hirata, Tanaka, Zeng, Hozumi, & Arai, 2006; Kalashnikova, Zueva Iu, Pugacheva, & Korsakova, 2004; Picard, Amado, Mouchet-Mages, Olie, & Krebs, 2008; Stout & Johnson, 2005)

In summary, the extra-pyramidal system is defined as a neural network involved in involuntary motor functions. The major components of this system are the BG and the

cerebellum. This system was thought to be involved in only motor functions and to be independent of pyramidal system. However, recent studies report that the extra-pyramidal system has connections with the pyramidal system and affects cognitive functions as well as motor functions.(Hirata, et al., 2006; Kalashnikova, et al., 2004; Picard, et al., 2008; Stout & Johnson, 2005)

II. Functional analysis of the SN

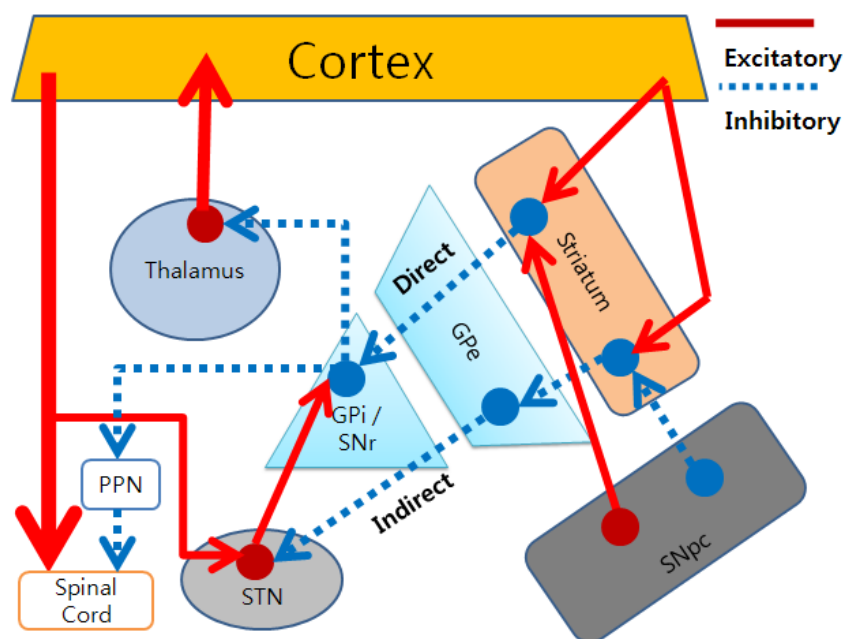


Figure 2. Circuit of Basal Ganglia

1. The SN in the intrinsic circuit of basal ganglia

The substantia nigra(SN) is a major element of the BG. Soemmerring named the SN, “black substance” in Latin, in 1792.(Druey, 1987; Moog & Karenberg, 2004; Rosler, 1980)

The sub-nuclei of SN are the substantia nigra pars compacta(SNpc), the substantia nigra pars reticulata(SNpr), and the substantia nigra lateralis. The border between the SNpc and the

SNpr is clear because the SNpc appears darker than the SNpr due to a rich density of dopaminergic(DA) cell bodies in the SNpc compared to low neuronal density in the SNpr which primarily composed of GABAergic neurons and axons of neurons into SNpc.(Deniau, Mailly, Maurice, & Charpier, 2007; Francois, Percheron, Yelnik, & Heyner, 1985; Halliday & Tork, 1986; Paxinos, 1995; Poirier, Giguere, & Marchand, 1983)

The SNpc and the SNpr play an important role in the function of the BG circuit. The BG transmits neocortical information to the output nuclei of BG through two major pathways: direct and indirect.(Kandel, et al., 2000; Squire, 2003b; Takakusaki, Saitoh, Harada, & Kashiwayanagi, 2004) The major input of the BG comes from the cerebral cortex and thalamus in quantitative terms and the main recipient of these innervations is the striatum.(S. N. Haber & Fudge, 1997; Halliday & Tork, 1986; Kandel, et al., 2000; Paxinos, 1995; Squire, 2003b) These projections are glutamatergic and then the striatum projects GABAergic neurons to BG output nuclei, the SNpr and the internal segment of globus pallidus(GPi), by both direct and indirect pathways. Through the indirect pathway, the striatum sends a projection first to the external segment of the globus pallidus(GPe) and from there to the SNpr/GPi via the subthalamic nucleus(STN). The direct pathway runs directly from the striatum to the SNpr/GPi. The GPe also sends an inhibitory projection to the SNpr/GPi same as to the STN but the most of projections from the GPe innervate the STN providing an excitatory glutamatergic projection to the SNpr/GPi. In contrast, the direct pathways suppress

the activation of the SNpr/GPi by an inhibitory GABAergic projection.(Hutchison, et al., 2004; Kandel, et al., 2000; Squire, 2003b; Tepper, Abercrombie, & Bolam, 2007) Thus, the direct pathways gives a positive feedback while the indirect pathways provides a negative feedback to BG output nuclei.

The main role of the SNpc in the BG circuit is the information modulator via the DA projection. Two DA projections from the SNpc to the striatum regulate the stream of thalamic and cortical information throughout the BG; the first DA projection facilitates the direct pathway, and the second DA projection inhibits the indirect pathway. This modulation causes state-dependent changes in the striatal neuronal excitability which affects the output of the BG from the SNpr/GPi. The SNpr, a major output nucleus of the BG, is the delivery center sending the cortical and thalamic information to various areas through GABAergic projections. The SNpr/GPi receives GABAergic projections from the striatum through two major pathways and glutamatergic projections from STN.(Deniau, et al., 2007; Kandel, et al., 2000; Takakusaki, Saitoh, et al., 2004) The SNpr/Gpi provides divergent projections to the superior colliculus(SC), the reticular formation(RF), the PPN, and the lateral habenula(HBN). In addition, the SNpr/GPi provide major feedback to the cerebral cortex via the thalamus: anterior cingulate area(ACA), dorsolateral prefrontal cortex (DLC), frontal eye fields (FEF), lateral orbitofrontal cortex (LOF), and somatosensory cortex (SMA).(S. N. Haber & Fudge, 1997; Halliday & Tork, 1986; Kandel, et al., 2000; Paxinos, 1995) These neural connections

functions affected by the modulation of the SNpc in the BG. (Galvan & Wichmann, 2007; Hutchison, et al., 2004; Kandel, et al., 2000) The motor and cognitive deficits due to the degeneration of the SNpc DA neuron support that the DA projections of the SNpc effect various brain functions in PD. This model, based on human disease, may not perfectly to explain the normal function of the SN. However, the PD model based on human disease is necessary to develop testable hypotheses and new treatment.(DeLong & Wichmann, 2007; Foley & Riederer, 2000; Linazasoro & van Blercom, 2006; Nau, 2005; Ogura, Nakao, Nakai, Uematsu, & Itakura, 2004; Rektor, et al., 2004; Slaght, et al., 2002; Sobstyl, Zabek, Koziara, & Kadziolka, 2003; Squire, 2003a) The relationship between symptoms of PD and neuronal circuits is essential to discuss the structure and function of the SN.

In summary, the SN plays a crucial role in the intrinsic circuit of BG. The SNpc is the regulator and the SNpr is the distributor in the flow of information. These functions of the SN in the intrinsic circuit of BG influence many brain functions through extrinsic BG circuits such as cortical cortico-basal ganglia circuits and basal ganglia-brainstem(BG-BS) system. The topic of following section is about brain functions related to the SN through extrinsic BG circuits and system.

2. The SN in the extrinsic circuit of basal ganglia

The SN plays an important role in brain functions through cortico-basal ganglia circuits and basal ganglia-brainstem(BG-BS) system. There are five parallel segregated cortico-basal ganglia circuits named for the following cortical target areas: motor, oculomotor, dorsolateral prefrontal, lateral orbitofrontal, and anterior cingulated circuits.(Squire, 2003b)

The functions involved by the target cortex area of each circuit are associated with functional regulation of the SN. In addition to multiple cortico-basal ganglia loops, the regulation of the SN can effect a BG-BS system involving in control of postural muscle tone, locomotion, and REM sleep.(Takakusaki, Oohinata-Sugimoto, Saitoh, & Habaguchi, 2004; Takakusaki, Saitoh, et al., 2004) In the BG-BS system, the SNpr/GPi projects inhibitory GABA-ergic neurons to the SC, the RF, the PPN and the HBN.(Takakusaki, Saitoh, et al., 2004) Through these projections in the BG-BS system and the cortico-basal ganglia circuits, the SN affects diverse brain functions.

The SN plays a crucial role in motor as a critical component of the motor cortico-basal ganglia circuit. The motor cortico-basal ganglia circuit connects the BG, thalamus and cerebral cortex; especially both caudal and rostral cortical motor areas projecting topographically to the striatum.(Aosaki, et al., 1994; Flaherty & Graybiel, 1994; S. N. Haber, 2003; S. N. Haber & Fudge, 1997; S. N. Haber, Fudge, & McFarland, 2000; Kemp & Powell, 1970; Kunzle, 1975, 1978) Caudal motor areas consist of motor cortex(M1), SMA, caudal

cingulate motor area (CMAc), and caudal premotor area (PM) areas which are involved with motor execution and provide descending projections directly to spinal motor nuclei. Rostral motor areas project to caudal motor area, including PreSMA, CMAr and rostral PM areas, are involved in sequence generation, and motor learning. (S. N. Haber, 2003) In this motor circuit, the SMA receives the feedback from the SNpr/GPi via the thalamus. The SNpc regulates motor function through this motor circuit. Therefore, degeneration of SNpc DA neurons in PD causes voluntary movement difficulties. The SN modulation for voluntary motor function is supported by voluntary motor deficits due to the degeneration of SNpc. In addition, the SN is involved in other motor functions by the DA modulation.

The SN, in addition to voluntary motor function, is involved in involuntary motor functions, such as locomotion, postural muscle tone, and saccadic eye movements, through the BG-BS system. (O. Hikosaka, Takikawa, & Kawagoe, 2000; Takakusaki, Oohinata-Sugimoto, et al., 2004; Takakusaki, Saitoh, et al., 2004) In the rat, the inhibitory GABAergic projections arising from the SNpr go to the midbrain locomotor region (MLR) (Garcia-Rill, 1997; Rossignol, 1996; Takakusaki, Habaguchi, Ohtinata-Sugimoto, Saitoh, & Sakamoto, 2003) and the PPN. (Lai & Siegel, 1990; Takakusaki, et al., 2003) The central pattern generator (CPG) receives the excitatory projection from the MLR via the locus coeruleus/raphe nuclei (LC/RN) and the medullary reticulospinal neuron (RSN). The PPN sends the excitatory acetylcholinergic projection to the muscle tone inhibitory system passing

through the pontin reticular formation (PRF) and the medullary RSN.(Takakusaki, Saitoh, et al., 2004) In the rat, stimulation of SNpr stops locomotion and delays the onset of locomotion, which means the SN effects both the dynamic and the steady state in locomotion.(Takakusaki, Saitoh, et al., 2004) In the human model, the increased output from the SNr can suppress the MLR to reduce walking velocity and can raise the muscle tone by reducing the activation of the PPN. (Goldman, Baty, Buckles, Sahrman, & Morris, 1998) Through the BS-BG system, the SN plays crucial role in locomotion and muscle tone control.

The SN plays an important role in the visual system to select appropriate signals to make a saccade to an object purposefully.(O. Hikosaka, et al., 2000; Squire, 2003b) Saccade is the series of involuntary, abrupt, rapid, small movements or jerks of both eyes simultaneously in changing the point of fixation.(Dorland, 2003) A saccade is essential function to screening the environment visually. The excitatory projection arises from the cerebral cortex and the inhibitory projection from the SNr innervate to the SC. The SN modulate a saccade purposefully by the direct pathway disinhibiting the SC and by the indirect pathways inhibiting SC. The model of PD supports this neural connection. The hyperactive BG output from the SNpr in PD suppresses activation of the SC.(O. Hikosaka, et al., 2000) Thus, PD patients have difficulties in performance of voluntary saccadic eye movement including anti-, memory-guided, and prospective saccades.(T. Crawford, Goodrich, Henderson, & Kennard, 1989; T. J. Crawford, Henderson, & Kennard, 1989; Crevits & De

Ridder, 1997; Crevits, Vandierendonck, Stuyven, Verschaete, & Wildenbeest, 2004; Kitagawa, Fukushima, & Tashiro, 1994; Lueck, Tanyeri, Crawford, Henderson, & Kennard, 1990; Muller, Wenger, Fertl, & Auff, 1994; Vermersch, et al., 1994; Vidailhet, et al., 1994) The patients demonstrate less ability to anticipate target steps in predictive and memory-guided tasks, and saccades were significantly hypometric but reflective saccades were not. (Blekher, Siemers, Abel, & Yee, 2000; Crevits, et al., 2004) In addition to the BG-SC connection, oculomotor and dorsolateral prefrontal cortico-basal ganglia circuits might be correspond to these voluntary saccades.³⁵

The SN is involved with cognitive functions, such as working-memory(WM),(Da Cunha, Angelucci, Canteras, Wonnacott, & Takahashi, 2002; Da Cunha, et al., 2003; Hotson & Boman, 1991; Levin, Briggs, Christopher, & Auman, 1994; Routtenberg & Holzman, 1973; Wichmann & Kliem, 2004) strategic planning,(Desmurget, Grafton, Vindras, Grea, & Turner, 2004; Houk & Wise, 1995; Monchi, Petrides, Strafella, Worsley, & Doyon, 2006; Schad, et al., 1992) and reward-based learning(Da Cunha, et al., 2006; Exner, Koschack, & Irle, 2002; Murray, et al., 2007; Packard & Knowlton, 2002; Sato & Hikosaka, 2002; Seger, 2006; Tait & Brown, 2008) through cortico-basal ganglia circuits. The DLC, the LOF, and the ACA receive the feedback from the SNpr via the thalamus through cortico-basal ganglia circuits and play a crucial role in cognitive functions.(Alexander, DeLong, & Strick, 1986; Middleton & Strick, 2002; Squire, 2003b) The main function of the DLC is executive functions including WM, set

shifting, and strategic planning.(Fuster, 2000, 2001; Goldman-Rakic, 1996; Passingham, 1993; E. E. Smith & Jonides, 1997) Brain imaging studies support the DLG works with the BG in matching and reasoning tasks.(Melrose, Poulin, & Stern, 2007) The orbital prefrontal cortex, closely connected to the medial prefrontal cortex, plays a crucial role in the development of goal-directed behaviors and reward-based learning.(Baxter, Parker, Lindner, Izquierdo, & Murray, 2000; Benevento, Fallon, Davis, & Rezak, 1977; Butner & Snyder, 1972; K. Hikosaka & Watanabe, 2000; Meunier, Bachevalier, & Mishkin, 1997; Rolls & Baylis, 1994; Schultz, Tremblay, & Hollerman, 2000) Patients with lesions of the orbital and medial prefrontal area have deficits in goal-directed behaviors and demonstrate socially inappropriate and impulsive behaviors.(Cummings, 1995; Eslinger & Damasio, 1985; Filley, 2001; Fuster, 1997b; Rolls, Burton, & Mora, 1980) The ACC contribute to functions such as regulation of emotional response, error detection, anticipation of tasks, and motivation.(Bush, et al., 1999; Nieuwenhuis, Ridderinkhof, Blom, Band, & Kok, 2001; Pineda, et al., 1998) The SN regulate the signals from these cortices for various cognitive functions through DLG, LOF, and ACC cortico-basal ganglia circuits.

The SN appears to play a role in REM sleep, arousal state, and attention by modulating the PPN and the ascending reticular activation system(ARAS). The BG GABAergic output modulates the ARAS through two systems: the direct nigrothalamic projection and the PPN projection. The SNpr gives GABAergic projection to the thalamus

and the PPN. The DA neurons in the SNpr have connections with the cholinergic and non-cholinergic excitatory neurons in the PPN.(Kitai, 1998; Takakusaki, Shiroyama, Yamamoto, & Kitai, 1996) These neural connections between the SN and the PPN are supported by both electrophysiological(Grace & Bunney, 1979; Hausser & Yung, 1994; Paladini, Celada, & Tepper, 1999; Saitoh, Isa, & Takakusaki, 2004) and neuroanatomical(W. J. Nauta, Smith, Faull, & Domesick, 1978; Y. Smith & Bolam, 1990; von Krosigk, Smith, Bolam, & Smith, 1992) studies. The activities of the PPN are involved in the cognitive functions, including arousal levels, execution and preparation of movement, the level of task performance, and reward.(Kobayashi, Inoue, Yamamoto, Isa, & Aizawa, 2002) The PPN is also regarded as an integrative interface between the numerous signals needed to perform purposeful behaviors.(Kobayashi, Inoue, & Isa, 2004) In the BG-BS system related to such cognitive functions, the SNpr provides the inhibitory GABAergic projection to the PPN to integrate information related to reward and reinforcement by modulating the excitability of DA and cholinergic neurons.(Takakusaki, Saitoh, et al., 2004) The SNpr controls the activation of the PPN to manipulate the various cognitive functions by regulating the direct and the indirect pathways through the SNpc.

Animal models support that the SN is involved in both motor and cognitive functions related to target cortical areas of the cortico-basal ganglia circuit and the BG-BS system. In animal models, such as the rat and the monkey, the neural connections of the SN with other

brain areas established by electrophysiological and neurochemical studies.(S. N. Haber, 2003; Takakusaki, Oohinata-Sugimoto, et al., 2004; Takakusaki, Saitoh, et al., 2004) Based on the knowledge of neural networks with the SN from animal studies, the pathological animal model has been developed. Neurotoxin, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), can generate artificial neurodegeneration in nigrostriatal DA system.(Arai, Misugi, Goshima, & Misu, 1990; Chang, Wang, Lu, & Lee, 1993) Degeneration in the system induces the imbalance between the direct and indirect pathways and then the excitability of the BG output from the SNpr/GPi increases. Increased BG output suppresses the PPN and decreases feedback to diverse cerebral cortices via the thalamus. This abnormal output signal suppressed the activity of the thalamus and other nuclei in the BG-BS system such as the PPN, the MLR, and the SC because the SNpr gives inhibitory GABAergic projections to these nuclei. Therefore, the symptoms of animal PD model, such as hypokinesia, depression, WM deficit, and eye movement deficit, are believed to be related to SN functions. Also electrical stimulation of the SNpr produced gait deficit in the rat, and changed REM sleep in the cat.(Takakusaki, Saitoh, et al., 2004) These animal models provide evidence for the neural network and the function of the SN.

In summary, the SN influences brain functions through the extrinsic BG circuits. Various brain areas have connections with the BG through the extrinsic BG circuits such as cortico-basal ganglia circuits and the BG-BS system. Many studies in animal models support

these neural connections and brain functions the SN involves in. The next section is about evidence of brain functions related to the SN in human disease model.

3. The functional deficits of the SN in human

The symptoms in PD supports that the SN functions related to the neural networks with other brain areas. PD patients show a ranged motor and cognitive deficit. The primary motor symptom of PD is bradykinesia, characterized by muscle rigidity, tremor, and a slowing of physical movement. For example, the movement time increased and gait velocity decreased significantly in PD patients without dementia.(Goldman, et al., 1998) These motor deficits are related to the cortico-basal ganglia motor circuit involved in motor control and to the PPN-CPG playing a role in gait pattern generation.(Takakusaki, Saitoh, et al., 2004) The cognitive deficits also support the neural networks the SN involves in. PD patients experience frontal dysfunction characterized by deficits in WM, visuospatial function, and executive function because abnormal hyperactive BG output signal to suppress the thalamus provide insufficient feedback to the DLC through the dorsolateral prefrontal cortico-basal ganglia circuit.(Karamat, Ilmberger, Poewe, & Gerstenbrand, 1991; Muslimovic, Post, Speelman, & Schmand, 2005; Vera-Cuesta, Vera-Acosta, Alvarez-Gonzalez, Fernandez-Maderos, & Casabona-Fernandez, 2006; Zgaljardic, et al., 2007) Other cognitive dysfunctions in PD such as apathy, impaired social and emotional behavior, and impulsive behavior are mainly related to the mesocotilimbic system, but these symptoms can be affected by SNpc DA neurons

through the LOF and the ACA cortico-basal ganglia circuits. In addition, sleep disorder is evidence of the SN function in the BG-BS system. About 90% of patients with Parkinsonism have sleep disorders such as daytime sleepiness, sleep-related breathing disorders and parasomnias.(Bliwise, Willians, Irbe, Ansari, & Rye, 2000; Eisensehr, v Lindeiner, Jager, & Noachtar, 2001; Szucs, Kovacs, Lalit, & Peter, 2007; Vendette, et al., 2007) REM sleep behavior disorder(RBD)(Culebras & Moore, 1989; Sanford, et al., 1994), Parasomnia reduces or abolishes muscle paralysis that normally occurs during REM sleep, which is associated with the BG-BS system, specifically GABA-ergic projection from the SNpr to the PPN. The symptom of PD is the strong evidence supporting the SN is one of the most important components within neural networks for both cognitive and motor function.

4. Summary of the SN function

The SN plays a crucial role in motor and cognitive functions through cortico-basal ganglia circuits and the BG-BS system. The SN can be divided by two parts, the SNpc and the SNpr, anatomically, physiologically, and functionally. The SNpc modulates flow of information in the BG by balancing the direct and indirect pathways through the DA projection and the SNpr provides BG output mainly by GABAergic projections to the thalamus and the brain stem. Both of them are components of the cortico-basal ganglia circuit providing BG feedback to various cortical areas and the BG-BS system projecting to numerous nuclei in the BS. Many studies in human and animal model provides evidence

supporting the concept that the SN, through cortico-basal ganglia circuits and the BG-BS system, is involved in numerous brain functions including motor control, executive function, WF, and sleep.

III. Functional analysis of the VTA

1. Connections of the VTA with other brain areas

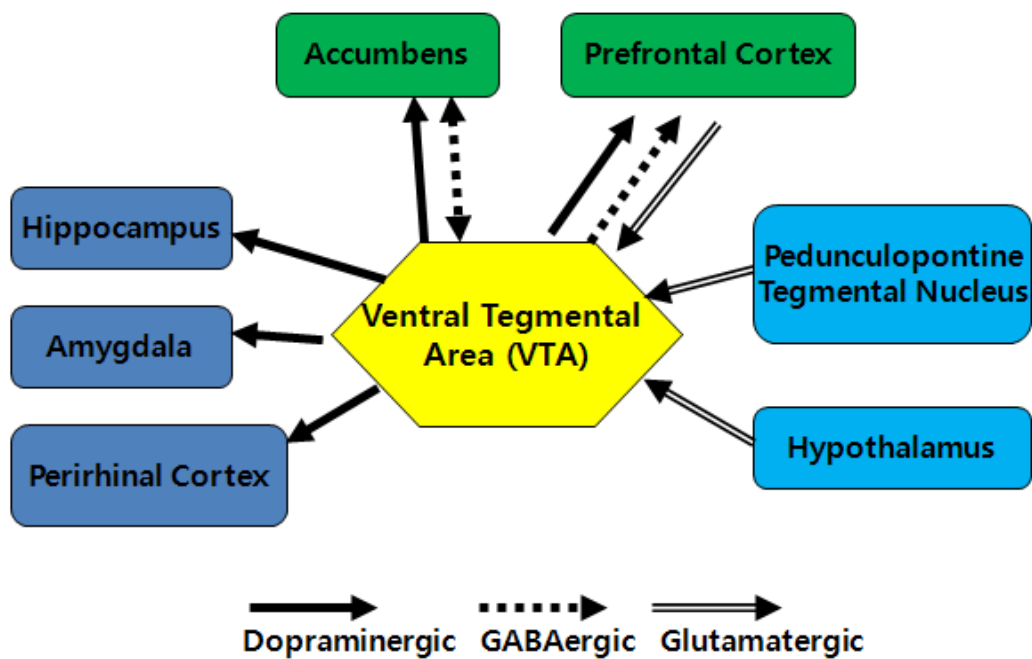


Figure 4. VTA projections with other brain area

The VTA, located lateral to the SNpc and the medial lemniscus, posterior to the retrorubral field, and dorsal to the red nucleus, is a major component of the midbrain DA system and is comprised of two main pathways. The first is the mesolimbic pathway to the nucleus accumbens(ANc) and the second is the mesocortical pathway to the cortical area of the frontal lobe. In addition to two main pathways, dopaminergic neurons in the VTA project

to various areas including amygdala and hippocampus.(Korotkova, Ponomarenko, Brown, & Haas, 2004; Oades & Halliday, 1987; Paxinos, 1995) Thus, the depletion of dopaminergic neurons in the VTA cause diverse dysfunctions related to the areas connected with the VTA.(Bosboom, Stoffers, & Wolters, 2004; Lieberman, 2006) The VTA is involved with various brain functions through neural networks mainly by DA projections.

The neurons of the VTA project to diverse areas in the human brain. Several types of neurons are in the VTA but DA projections comprise approximately 95% of all projections.(Paxinos, 1995) DA neurons project to the prefrontal cortex, the hippocampus, the amygdala, the NAc, and olfactory tubercles in the human brain. The NAc receives both dopaminergic and nondopaminergic projections.(Korotkova, et al., 2004; Paxinos, 1995) While most outputs of the VTA are dopaminergic, all inputs to the VTA are non-dopaminergic. The inputs, containing GABAergic, and glutamatergic, come from the subthalamic nucleus, the amygdala, the prefrontal cortex, and the NAc.(Korotkova, et al., 2004; Oades & Halliday, 1987; Paxinos, 1995)

In summary, the VTA has connections with various brain areas. DA projection is majority of the VTA output to other areas such as the ANc, the amygdale, the prefrontal cortex, and the hippocampus. The VTA involves in brain functions through DA projections to various brain areas. The topic of the next section is brain functions related to the VTA.

2. The brain functions related to the VTA

The VTA is involved with reasoning and planning through DA projection to some subdivisions of the prefrontal area, such as the medial, dorsolateral, and orbital cortices.(Kandel, et al., 2000; Lundy-Ekman, 2002; Squire, 2003b) The prefrontal area is widely believed to a critical structure for most complicated cognitive function such as reasoning, planning, and executive function. Some subdivisions of the prefrontal area, such as the medial, dorsolateral, and orbital cortices, have connections with the VTA.(Kandel, et al., 2000; Lundy-Ekman, 2002; Squire, 2003b) Specifically the DLC is an important part of the neural network contributing WM involving to maintain a short-term storage and to process or manipulate information held in WM “buffer” called the “central executive”.(Fuster, 1997a; Levy & Goldman-Rakic, 2000; Shallice, 1982; E. E. Smith & Jonides, 1999) The prefrontal cortex were reported to mediate the firing pattern of DA neurons in the VTA(Gariano & Groves, 1988; Murase, Grenhoff, Chouvet, Gonon, & Svensson, 1993; Overton, Tong, & Clark, 1996; Svensson & Tung, 1989; Tong, Overton, & Clark, 1996). The stimulation on the prefrontal cortex increases the activity of DA neurons in the VTA and induces action potential-dependent DA release.(Gao, et al., 2007; Gonon, 1988) The VTA modulates the temporal code by transmitters including DA in medial prefrontal neurons projecting the mediodorsal thalamus and the NAc which are the primary sites for short-term WM.(Au-Young, Shen, & Yang, 1999; Floresco, Seamans, & Phillips, 1997; Kuroda, Murakami,

Igarashi, & Okada, 1996) This reciprocal mechanism and functional coupling between the prefrontal cortex and the VTA strongly supports that the VTA has critical function in WM and executive function. Deficits of executive function and WM in human with the degeneration of DA neurons in the VTA support the role of the VTA in WM and executive function. (Cooper & Sagar, 1993; Cooper, et al., 1992; Owen, et al., 1993)

The VTA has an influence on the function of the hippocampus through DA projections. The hippocampus, a structure located at the medial temporal lobe, is involved in memory and spatial navigation. In memory, the hippocampus has a major role in developing new memories, such as episodic or autobiographic memory.(Kandel, et al., 2000; Riedel & Micheau, 2001; Squire, 2003b) The hippocampus comprises a large part of the temporal memory system retaining the memory after phase consolidation. Processing and storing spatial information is also another crucial role of the hippocampus. In rats, specific neurons called “place cells” fire when animals find themselves in a certain place.(Frank, Brown, & Stanley, 2006) A brain imaging study reveals taxi drivers have bigger hippocampi than control drivers. More experienced drivers have a bigger hippocampus.(Maguire, et al., 2000) The VTA forms the functional loop with the hippocampus to mediate the registration of information into long-term memory.(Lisman & Grace, 2005) When the new information arrives in the hippocampus, the hippocampal-VTA loop begins to activate. Then the resulting signal runs into the VTA through the subiculum, the NAc, and ventral pallidum. The

novelty-dependent neurons in the VTA fire to release DA within the hippocampus in the upward arm of the functional loop.(Lisman & Grace, 2005) This process in the hippocampal-VTA loop enhances the long-term memory and learning. In animal model, the D1 antagonist blocked completely the late phase of long-term memory.(Bach, et al., 1999; Frey, Huang, & Kandel, 1993; Frey, Matthies, Reymann, & Matthies, 1991; Frey, Schroeder, & Matthies, 1990; Huang & Kandel, 1995) Conversely the D1 agonist enhanced the late long-term memory.(Swanson-Park, et al., 1999) In human model, DA neuron degeneration in VTA induces the mnemonic dysfunction of the deficit in explicit memory.(Buytenhuijs, et al., 1994; Taylor, Saint-Cyr, & Lang, 1990) With defective explicit memory, PD patients have difficulty accessing stored information, which is usually remedied by semantic cueing or probing.(Pillon, Deweer, Agid, & Dubois, 1993) Both animal and human models provide evidence for the function of the hippocampal-VTA loop.

The VTA, having reciprocal connections with the NAc, takes the major role in reward, pleasure, and addiction.(Kandel, et al., 2000; Squire, 2003b) DA inputs from the VTA via the mesolimbic pathway are thought to regulate the activation of neurons within the NAc located at the head of the caudate and the anterior portion of the putamen.(Deadwyler, Hayashizaki, Cheer, & Hampson, 2004) These terminals related to the action of highly-addictive drugs increasing to dopamine level in the NAc.(Deadwyler, et al., 2004; Kandel, et al., 2000; Lundy-Ekman, 2002) The stimulation of DA neuron in the NAc induces

addictive behavior such as gambling and shopping by the additional high-dose dopaminergic drug treatment. In PD with the DA neurons degeneration of the VTA, the patient showed the episode of pathologic gambling after increasing dose of L-Dopa.(Gschwandtner, Aston, Renaud, & Fuhr, 2001) Lack of the dopaminergic stimulation, due to the degeneration of DA neurons, also enhances reward-seeking behavior to compensate the loss of internal cues.(Gschwandtner, et al., 2001) In various animal models, repeated exposure to opiates enhanced the behavioral effect referred to as sensitization.(Spanagel, 1995; Spanagel, Almeida, & Shippenberg, 1993) These behavioral changes are related to the DA projections of mesolimbic pathways mainly from the VTA to the NAc.(Kalivas & Duffy, 1993a, 1993b; Weiss, Paulus, Lorang, & Koob, 1992) Recent human brain imaging studies also provide evidence that supports the VTA's role in the reward seeking behavior.(D'Ardenne, McClure, Nystrom, & Cohen, 2008; Wittmann, Schiltz, Boehler, & Duzel, 2008) The positive prediction error was significantly related to blood oxygen level-dependent (BOLD) response of the VTA.(D'Ardenne, et al., 2008) The SN/VTA activated during reward prediction. The contrast between neural cue and rewarding-prediction showed significant activation of the SN/VTA.(Wittmann, et al., 2008) The VTA is an important component with the ANc in reward and pleasure, which are supported by behavioral and brain imaging studies in human and animal models.

The VTA is involved with regulating emotion, motivation, and emotional association

with memory, by mutual connection with the amygdala.(Kandel, et al., 2000; Lundy-Ekman, 2002; Squire, 2003b) The amygdala is associated with emotional learning and memory such as Pavlovian fear conditioning which is making a conditioned stimulus (usually a beep sound) paired with noxious unconditioned stimulus (usually electrical shock).(Ferry, Roozendaal, & McGaugh, 1999; Maren, 2001) The amygdala projects to various brain areas; the hypothalamus for the regulation of the sympathetic nervous system, the reticular nucleus, and the nuclei of the trigeminal nerve and facial nerve for expressions of fear.(Maren, 2001) The amygdala is also thought to be an important part in the modulation of memory consolidation; a process in which the instant memory is slowly absorbed into long-term storage.(Ferry, et al., 1999) During the modulation, the emotional arousal after the learning event facilitates memory consolidation. The correlation between amygdalar activity and retention for information depends on the relative “emotionalness” of information. The activation of the amygdala increasing with more emotionally-arousing information positively correlates to retention.(Ferry, et al., 1999; Maren, 2001; Pell & Leonard, 2005; Yoshimura, Kawamura, Masaoka, & Homma, 2005) Intra-VTA injections of morphine impaired long-term memory retention in rats after passive avoidance training with electrical shock.(Zarrindast, Farajzadeh, Rostami, Rezayof, & Nourjah, 2005) The opiates are reported to suppress GABA inhibitory input to DA neurons in the VTA, in this manner expanding DA release.(Harris, Wimmer, Byrne, & Aston-Jones, 2004; S. W. Johnson & North, 1992) This

abnormal response to morphine in the VTA DA system affects memory retention and learning, which are primary functions of amygdala, by mutual connections between the VTA and amygdala.

In summary, the VTA involves in various brain functions through neural connections. Many studies in animal models, such as the rat, provide evidence of these neural networks and brain functions. The topic of the next section is the symptoms of VTA deficits supporting the neural connections and brain functions in human disease model.

3. The VTA dysfunction in human

The VTA has a crucial role in emotion through the mesolimbic pathway. A number of recent studies reported that the DA system of the VTA in the mesolimbic pathway cause pathology and must be targeted for treatment of depression.(Dunlop & Nemeroff, 2007; Gershon, Vishne, & Grunhaus, 2007) In the animal model, Flinders sensitive line(FSL) rats (an animal model for depression) exhibited decreased firing variability in VTA DA neurons(Friedman, et al., 2005) and reduced dopamine level in the NAc.(Friedman, et al., 2007) Pharmacological treatments for depression with desipramine increased the dopaminergic mesolimbic activity and DA level.(Friedman, Friedman, Dremencov, & Yadid, 2008) In human model, the loss of dopaminergic neurons in the VTA effects the mesolimbic pathway which have been implicated in apathy, and depression.(Giovannoni, O'Sullivan, Turner, Manson, & Lees, 2000; Ikemoto & Panksepp, 1999; Mann & Kapur, 1995; Willner,

1997) According to a review of 45 studies published from 1992 to 1998, about 31% of PD patients were suffering from depression.(Lieberman, 2006) The VTA is a critical structure in the mesolimbic pathway in reaction to emotion.

Human PD model provides evidence for function and the networking of the VTA. PD patients have several deficits related to DA neuron degeneration in the VTA such as visuospatial dysfunction, facial expression decoding, and pathologic gambling.(Driver-Dunckley, Samanta, & Stacy, 2003; Gschwandtner, et al., 2001; Kurlan, 2004; Molina, et al., 2000; Pell & Leonard, 2005; Yoshimura, et al., 2005) The hippocampus is involved in spatial information, the amygdala in recognizing facial expressions, and the NAc, related to addictive behaviors, receive dopaminergic projections from the VTA.(Korotkova, et al., 2004; Oades & Halliday, 1987; Paxinos, 1995) Thus, the loss of DA neurons in the VTA could cause visuospatial dysfunction, facial expression decoding, and pathological gambling in PD. These symptoms provide positive evidence support the brain functions related to the VTA neurons.

4. Summary of the VTA functions

The VTA plays a critical role in diverse brain functions, such as executive function, WM, emotion, and reward prediction, through various connections and functional loops with other brain areas including the prefrontal area, the hippocampus, the NAc, and the amygdala. These functions and neural networks of the VTA are supported by animal and human models.

IV. Comparison of non-human primate and rodent models

1. Introduction

Various animal models (i.e., rat, monkey) have been used in researching on the SNpc and the VTA. Vicq d'Azyr first identified the SN in 1786,(Vicq D'Azyr, 1786) and Tsai first described the VTA as a morphologically distinct body in 1925.(Tasai, 1925) Following these identifications, the relationship between the SN and the motor system was revealed when several investigators found the link between the SN and PD in 19th century.(Bremer, 1920; Brissaud, 1895) In 1940s, the VTA was defined in various species, including armadillo, dog, and cat.(Fox, 1941; Papez, 1932; Rioch, 1929) In the early 1950, the hypothesis that the DA neurons in the midbrain, especially in the VTA, are associated with psychosis and behavioral disorders.(Baldessarini, 1985) In the SNpc and the VTA, the degeneration of DA neurons causes a constellation of motor and cognitive symptoms in PD, which raises the question about the role of the DA midbrain neurons. The various studies on the roles of DA midbrain neurons, especially the SNpc and the VTA, were based on work in the rat and the monkey.(S. N. Haber & Fudge, 1997)

Since the 1970s, the cytoarchitecture of the VTA has received considerable attention in two species: the rat(Phillipson, 1979a, 1979b) and the monkey(Felten, Laties, & Carpenter, 1974; Garver & Sladek, 1975; Hervonen, Tuohimaa, Kanerva, & Lietzen, 1974; Hubbard & Di Carlo, 1974). The studies based on work in the rat established five basic component nuclei

of the VTA: three medial (rostral linear nucleus, LR; central linear nucleus, LC; and interfascicular nucleus, IF) and two lateral nuclei (paranigral nucleus, PN; and parabrachial pigmented nucleus, PBP).(Phillipson, 1979a, 1979b) These major cytoarchitectonic components or the equivalent nuclei were also identified in the monkey(Felten, et al., 1974; Felten & Sladek, 1983; Garver & Sladek, 1975; Hubbard & Di Carlo, 1974; Schofield & Everitt, 1981; Tanaka, Ishikawa, & Shimada, 1982) and human(Bogerts, Hantsch, & Herzer, 1983; Halliday & Tork, 1986; Olszewski & Baxter, 1954). Thus, comparative anatomy of the VTA across primate and rodent is possible. Unbiased stereological methods have radically increased the accuracy of neuron counts, which facilitate cross-species analysis. The SN is less complex and easier to define across various species, especially the SNpc, because the colocalization of DA neurons with neuromelanin in the SNpc makes the SNpc look darker than the surrounding areas. The purpose of this chapter is to compare cytoarchitectures and morphologies of the SN and the VTA between the rat and the monkey.

2. Cytoarchitectural comparison of the SNpc

Species	Para	SNpc	VTA	PBP	PN	IF	LR	LC
Rat	V	0.3	1.2	0.6	0.3	0.1	0.1	0.2
	N	12,000	27,000	11,000	7,000	6,000	840	2,000
	D	561	309	254	343	604	210	199
	S	16	13	14	13	11	15	14
Monkey	V	6.3	6.5	4.5	0.7	0.2	0.3	0.8
	N	71,000	47,000	37,000	6,000	2,000	500	2,000
	D	260	80	147	133	103	27	50
	S	23	16	18	16	14	15	17
Human	V	68	183	101	33	4	9	37
	N	436,000	690,000	288,000	256,000	46,000	17	83,000
	D	200	120	86	185	238	61	68
	S	31	26	30	24	21	31	30

Table 1. Cytoarchitecture of subnuclei in the SN and VTA cross species.

Para, parameter; SNpc, substantia nigra; VTA, ventral tegmental area; PBP, parabrachial pigmented nucleus; PN, paranigral nucleus; IF, interfascicular nucleus; LR, rostral linear nucleus; LC, central linear nucleus; V, volume(mm^3); N, number; D, density(cells/mm^2); S, soma size(μm). (modified from Halliday (Halliday & Tork, 1986))

The SNpc of the rat is 0.3mm^3 in volume and about 10,000 to 12,000 neurons on each side. The recent stereological study reported the number of TH+ DA neurons is about 4,000 on each side in the SNpc. (Scott, Diaz, & Ahmad, 2007) It consists of the agglomeration of cells shaping a band overlying the cerebral peduncles. The density of the SNpc is 561 cells/mm^2 in the rat. The average of soma diameter is $16\pm 4\mu\text{m}$ (mean \pm SD) and the range is 6 to $33\mu\text{m}$. (Halliday & Tork, 1986) Three groups of SNpc neurons were identified in the rat. In the ventral region of the SNpc, the first type of neurons gives the shape of upside-down pyramidal neurons of the cortex and the dendrites often invade the SNpr. (Paxinos, 1995) However, the second type of neuron is located at the dorsal aspect of SNpc and the dendrites do not run into the SNpr. (Bjorklund & Lindvall, 1975) The second group has radiating

medio-lateral dendrites. The third neuron is a small stellate neuron, a nondopaminergic interneuron comprising 10% of SNpc neurons.(Paxinos, 1995) While the majority of the SNpc neurons are DA neurons, the SNpr neurons are primarily multi-polar GABAergic and ovoid, round or triangular in shape.(Gulley & Wood, 1971; Juraska, Wilson, & Groves, 1977; Poirier, et al., 1983) The soma size is approximately 20 X 45 μm and the medium size DA neurons are scattered in the SNpr beside the typical GABA-ergic neurons.(Paxinos, 1995)

The SNpc of the Pigtailed Macaque (*Macaca nemestrina*) monkey forms distinct dark bands of cells dorsal to the cerebral peduncles. Commonly, the two bands of cells appear at about 45° to each other. The volume of the SNpc is 6.2 mm³ and contains about 71,000 cells in the monkey. The density of the SNpc is 260 cells/mm² and the diameter of soma is 23 $\mu\pm$ 5 μ (mean \pm SD).(Halliday & Tork, 1986) In the primate, the SNpc is divided into three groups: α (a ventral group), β (a densocellular region), and γ (a dorsal group).(Olszewski & Baxter, 1954) The densocellular region(β) is the main group located between the ventral group(α) and the dorsal group(γ). The dendrites of the group β run into the major part of the SNpr in the primate. The cells of dorsal group (γ) are arranged loosely but the ventral group (α) is packed tightly with cells. The dendrites of the dorsal group(γ), lying just dorsal to the densocellular region(β), are oriented mediolateral direction but do not invade the SNpc. The neurons in the dorsal group(γ) continue to the VTA. The ventral group(α) is densely packed with neurons and permeate deep into the SNpr in isolated group or finger-like expansion. The

SNpr is ventral to the SNpc and contains predominately GABA-ergic neurons, having relatively small and sparse cell population. The border between the SNpr and the SNpc is not clear due to the DA neurons of the SNpc invading the SNpr.(S. N. Haber & Fudge, 1997)

In the comparison between the rat and the Pigtailed Macaque (*Macaca nemestrina*) monkey in the SNpc, the volume in the monkey(6.2mm^3) is over 20 times greater than in the rat(0.3mm^3) while the number of neurons in the monkey(71,000) is just over 7 times more than in the rat(10,000). Thus, the density of the rat(561 cells/mm^2) is much higher than the monkey(260 cells/mm^2). The soma size of SNpc in monkey ($23\mu\pm 5\mu$) is larger than the rat($16\pm 4\mu\text{m}$). In the monkey, the border between the SNpc and the SNpr is not linear because DA neurons invade the SNpr while the border is linear in the rat.

3. Cytoarchitectural comparison of the VTA

The VTA of the rat, located ventromedial to the red nuclei, contains about 27,000 cells and is 1.2 mm^3 in volume. A recent stereological study showed that the number of DA TH+ neurons is approximately 6,000 in the VTA on each hemisphere.(Ahmad, Park, Stenho-Bittel, & Lau, 2008) Among the five nuclei of VTA, the PBP is the largest nucleus in the rat. The PBP also contains the largest populations of cells. The PBP and the PN occupy 73% of volume of the VTA. The cell density of the IF is the highest in the rat. The IF takes only 9% of the VTA in volume but it contains 23% of cell population in the VTA. The soma diameter in the VTA is $13\mu\pm 3\mu\text{m}$ (mean \pm SD) and range from 6μ to 26μ . The LR contains the largest

cell and has various types of cells in size and shape. The PBP and PN cells are fusiform but the orientations of cells are different between two areas in the rat.(Halliday & Tork, 1986)

The VTA in the Pigtailed Macaque (*Macaca nemestrina*) monkey has approximately 47,000 cells and occupies 6.4 mm³. The PBP is the largest nucleus and others nuclei are small in comparison, especially the IF and the LR taking only 4% of VTA volume in the monkey. The PBP is 69% of VTA in volume and contains majority of cell population in the monkey (77%). The LC(1%) and LR(5%), located at the midline of the VTA, have principally small number of cells. These midline nuclei also have lowest density, especially LR(27 cells/mm²), while the PBP has the highest density (147 cells/ mm²). The IF has also over the average density of 80 cells/ mm² in the VTA. The soma size in the monkey is 16±5µm(mean±SD). The cell orientation and soma size of nuclei are different among the VAT nuclei. The PBP cells are the biggest in the VTA and fusiform orientated mostly mediolaterally. The IF is made up of small round cells and the PN contains small multiform cells.(Halliday & Tork, 1986)

There are some cytoarchitectural similarities in the VTA between the rat and the *Macaca nemestrina*. The volume of VTA is about three times larger in the monkey(6.4 mm³) than in the rat(1.2 mm³) but the number of neurons is just less than two times greater in the monkey(47,000) than in the rat(27,000). Therefore, the density of the VTA is higher in the rat(309 cells/ mm²) than in the monkey(80 cells/ mm²). The cell size of the VTA in the

monkey($16\mu\pm 5\mu\text{m}$) is bigger than in the rat($13\mu\pm 3\mu\text{m}$). All the six subnuclei are defined in the rat and the monkey. The PBP is the largest nucleus in both species. In the rat, the PBP has larger percentage in volume (50%) than the number of cells(40%) but less percentage volume(69%) than the number(77%) in the monkey. The percentage volume are very similar among IF, LR, and LC in both species.(Halliday & Tork, 1986) However, the distributions of the density ratio to the average density are not similar. In the rat, the IF has the highest density($604\text{ cells}/\text{mm}^2$) but the PBP has the highest density in the monkey($147\text{ cells}/\text{mm}^2$).

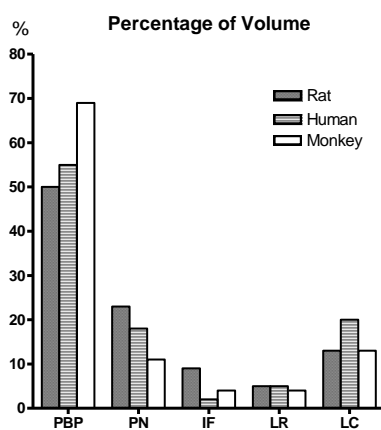


Figure 5. Volume of VTA subnuclei

(modified from Halliday(Halliday & Tork, 1986))

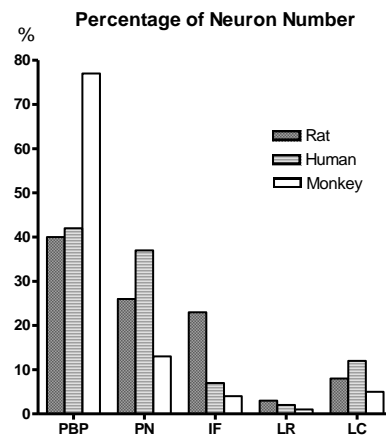


Figure 6. Neuron number of VTA subnuclei

(modified from Halliday(Halliday & Tork, 1986))

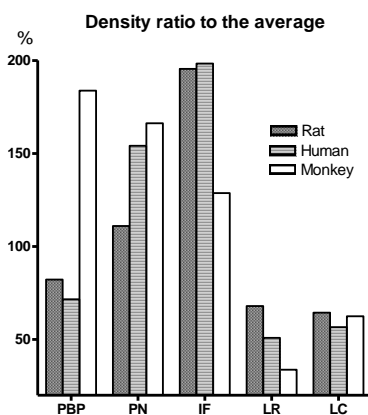


Figure 7. Density of VTA subnuclei

(modified from Halliday(Halliday & Tork, 1986))

4. Comparison of neural networks

The DA projections from the SNpc and the VTA in the rat innervate various brain regions.(Paxinos, 1995; van Domburg & ten Donkelaar, 1991) The striatum receives dense DA projections from the ventral and intermediate areas of the SNpc and ventro-lateral VTA. The striatum is composed of two different compartments named the patch and the matrix.(Goldman-Rakic, 1982) The striatal matrix has the DA innervations from dorsal tier neurons while the striatal patch has the DA innervations from ventral tier neurons.(Gerfen, Herkenham, & Thibault, 1987) The dorsal tier neurons project to the dorsolateral (sensorimotor) striatum. The DA projections, arising from the dorsal and middorsal VTA and medial SNpc, innervate ventral striatal structures including the NAc and olfactory tubercles. The VTA and the lateral SNpc give DA projections to the amygdala. The lateral septum receives DA projections from more ventral VTA neurons in the PN. Diverse cortices, including prefrontal, cingulate, perirhinal and entorhinal cortices, also take DA projections from the dorsal-most sheet of the VTA and the SNpc in the rat. Other areas, such as hippocampus, ventral pallidum and cerebellum, are reported to have sparse DA projections.(Paxinos, 1995)

The DA projections to the VTA and the SN in the rat arise from various areas such as the striatum, amygdala, cortices, etc.(Paxinos, 1995) The striatal matrix is the major input to GABA-ergic neurons in the SNpr while the striatal patches project to the ventral

SNpc.(Gerfen, 1992a, 1992b) The subthalamic nucleus gives the major excitatory input to GABAergic SNpr neurons.(Groenewegen & Berendse, 1990) The GABAergic projections to the SNpr and the SNpc arise from the globus pallidus and ventral pallidum.(S. N. Haber & Nauta, 1983; Kalivas, 1993; Y. Smith & Bolam, 1990, 1991; von Krosigk, et al., 1992) The VTA and the SNpc, particularly the dorsal-most tier, receive the projections from the amygdala,(Shinonaga, Takada, & Mizuno, 1992; Vankova, Arluison, Leviel, & Tramu, 1992; Wallace, Magnuson, & Gray, 1992) hypothalamus,(W. J. H. Nauta & Domesick, 1978; Wright, Tullock, & Arbuthnott, 1980) preoptic area, frontal cortex, and cingulate cortical areas(Hurley, Herbert, Moga, & Saper, 1991; Sesack & Pickel, 1992; Wyss & Sripanidkulchai, 1984) in the rat. These input and output in the VTA and the SN means that the VTA and the SN involved in related brain functions in the rat.

The VTA and the SN project DA to diverse brain area in the primate like in the rat. The striatum is the major target of the DA projections from the SN and VTA. Unlike in the rat, the dorsal tier does not innervate the striatal matrix and sensorimotor striatum. Although the research in the midbrain dopamine projection to cortex in the primate is not enough, the previous studies consistently reported that DA innervations from the VTA and the SN to cortices in the primate are much more widespread than in the rat.(Gaspar, Berger, Febvret, Vigny, & Henry, 1989; Gaspar, Duyckaerts, Alvarez, Javoy-Agid, & Berger, 1991; Goldman-Rakic & Brown, 1982; Levitt, Rakic, & Goldman-Rakic, 1984; Lewis, Campbell, Foote,

Goldstein, & Morrison, 1987; Lidow, 1995; Lidow, Goldman-Rakic, Gallager, & Rakic, 1991; Rosenberg & Lewis, 1995; Smiley, Williams, Szigeti, & Goldman-Rakic, 1992; Verney, Milosevic, Alvarez, & Berger, 1993; Williams & Goldman-Rakic, 1993) The DA projection arising from the midbrain innervates the prefrontal cortex (dorsal and orbitofrontal cortex), the parietal cortex, the temporal cortex and even to the occipital cortex. In addition, the DA projections to the hippocampus are from the VTA. The VTA and dorsal SNpc give DA projections to amygdala in the primate.(S. N. Haber & Fudge, 1997)

In the primate, the inputs to the VTA and SN come from diverse regions including the striatum, the amygdala, cortices and etc similar to the rat.(S. N. Haber & Fudge, 1997; van Domburg & ten Donkelaar, 1991) The striatum gives rise GABA-ergic projections massively back to the SN(Hedreen & DeLong, 1991; T. N. Johnson & Rosvold, 1971; Lynd-Balta & Haber, 1994; W. J. Nauta & Mehler, 1966; A. Parent, Bouchard, & Smith, 1984; A. Parent & Hazrati, 1994; Selemon & Goldman-Rakic, 1990; Y. Smith & Parent, 1986; Szabo, 1967, 1970) while the striatum send just few projections to the VTA.(S. N. Haber & Fudge, 1997)

In addition to the striatonigral connections, other afferent pathways have a connection with the DA neurons. The amygdala gives a rise to descending projections to the dorsal SNpc and the dorsocellular regions.(Olszewski & Baxter, 1954) Although the descending cortical projections are not clear, the projections from cortex to the SN have been reported.(Kunzle, 1978) The globus pallidus and the ventral pallidum give projections to the SN in the primate

similar to rats.(S. N. Haber, Lynd-Balta, & Mitchell, 1993) The dorsal raphe nucleus and the pedunculopontine also give descending projections to the VTA and the SN.(Lavoie & Parent, 1994) These neural networks in the VTA and the SN of the primate support that the VTA and the SN play a critical role in the various brain function related to the brain area connected with the VTA and SN.

The neural networks with the VTA and the SN are very similar between the rat and the primate. The common outputs from the VTA and the SN in both animals projects to the striatum, the hippocampus, the amygdala, and the prefrontal cortex. The common inputs in both animals come from the striatum, amygdala, and cortices. The similarity of the neural networks with the VTA and the SN means that the VTA and the SN are involved in the similar brain function in both animals. The VTA and SN have more connection with various cortex areas in the primate than in the rat.

5. Conclusion

Even though some differences in size and density exist between the rat and the monkey, the nuclear organization, neurotransmitters, and projections are similar. In the VTA and the SNpc, the number of neurons is much greater in the monkey than in the rat while the density is significantly higher in the rat. The VTA volume in the rat(1.2 mm^3) is bigger than the SNpc(0.3 mm^3) whereas the VTA volume in the monkey(6.5 mm^3) is similar to the SNpc(6.3 mm^3). Although the density and the size of cells are different, the relative positions

of subnuclei and the percentage volume of subnuclei are very similar in the rat and the monkey. The monkey has more connections between the VTA/SN and cortices than rat has. However, the afferent and efferent projections in the VTA and the SN are similar between rat and monkey in general.(S. N. Haber & Fudge, 1997; Halliday & Tork, 1986) The same kinds of neurotransmitters exist in the VTA/SN of the rat and the monkey: the major transmitters are DA and GABA.(S. N. Haber & Fudge, 1997; Halliday & Tork, 1986; Paxinos, 1995) In conclusion, there are anatomical, histological, neurochemical, and cytoarchitectural similarity between the rodent and the primate in the VTA and the SN.

V. Possibility of animal models for human translation in the SN and the VTA

1. Introduction

Many animal models have been used in the research on the SN and the VTA. Rat models are the most accepted model in research on the SN and the VTA, but the number of primate studies is increasing steadily.(S. N. Haber & Fudge, 1997) Although there are differences in anatomical structures between human and animal models, studies from rats and monkey have provide essential knowledge in understanding the pathways and roles of the SN and VTA in human brain. Some studies of animal models in the SN and the VTA provided evidence supporting that animal studies can be translated into human models. The evidence reveals that the some features of animal model are similar neurochemically and

cytoarchitecturally to the human model, and the symptoms of human PD model can be regenerated in the animal model. Thus, the animal model can be translated cautiously into the human model on the SN and the VTA. The purpose of this chapter is to discuss on possibility of animal model for human translation in the SN and the VTA.

2. Cytoarchitectural similarity

The similar cytoarchitectonic organization between human and other species is the basic evidence supporting that the animal models can be applied to human model in the SN and the VTA. The organization of cytoarchitectonic subdivisions in the SN and the VTA is very similar between human and animals, especially the rat and the monkey. Three subdivisions, such as the SNpc, the SNpr, and the pars lateralis, are generally recognized in the SN cross most mammalian species.(Halliday & Tork, 1986; Paxinos, 1995) In the primate and human, the SNpc invades into the SNpr in a finger-shape while it does not in the rat. In the SNpc, the DA neurons are denser than in SNpr and have neuromelanin, which makes the SNpc darker, although lower mammals contain less neuromelanin than nonhuman primates.(Bogerts, 1981; Herrero, et al., 1993; Marsden, 1983; McRitchie, Halliday, & Cartwright, 1995) In a study by Halliday and Tork(1986), cytoarchitectonic features of the VTA were similar among human, cat, rat and monkey. Five basic nuclei of the VTA were recognized in these four species. The cell number and size are different according to the body size cross species but the relative position of nuclei and cell distributions in the VTA are very

similar. Interestingly, the distribution of cells in the rat is more similar to in human than in the monkey,(Halliday & Tork, 1986) making it the ideal model for study.

The chemoarchitectural homogeneity of the SN and the VTA between human and other mammals supports human translation from animal model especially rodent and primate. The human SNpc has dense DA neurons, TH-positive, same as rat and monkey, and the SNpr contains GABA-ergic neurons cross species.(Bazelon, Fenichel, & Randall, 1967; A.M. Graybiel, 1986; Gulley & Wood, 1971; Juraska, et al., 1977; Mugnaini & Oertel, 1985; Andr e Parent, 1986; Poirier, et al., 1983) The SNpr of human and nonhuman primates also receive GABA-ergic projections mainly from the striatum, as similar to the rat. The substance-p-containing and enkephalin-ergic axons are commonly detected in the SNpr of these species.(Beach & McGeer, 1984; Bouras, Taban, & Constantinidis, 1984; Cuello & Kanazawa, 1978; Del Fiacco, Dessi, & Levanti, 1984; Gaspar, et al., 1983; Grafe, Forno, & Eng, 1985; A. M. Graybiel & Elde, 1983; S. Haber & Elde, 1982; S. N. Haber & Groenewegen, 1989; Inagaki, Kubota, & Kito, 1986; Inagaki & Parent, 1984; Mai, Stephens, Hopf, & Cuello, 1986; Pioro, Hughes, & Cuello, 1984; Waters, Peck, Rossor, Reynolds, & Hunt, 1988; Zech & Bogerts, 1985) The highest densities of DA D1 receptors in human brain are observed in the SN and other areas such as the caudate, the putamen, nucleus accumbens, the olfactory tubercle, and the medial part of the globus pallidus. In contrast to the SN, the VTA contains very low density of D1 receptors in human brain.(Cortes, Gueye, Pazos, Probst,

& Palacios, 1989) The distribution of D1 receptors in the rat brain is very similar to the human brain.(Dawson, Barone, Sidhu, Wamsley, & Chase, 1988; Savasta, Dubois, & Scatton, 1986) In addition to the D1 receptor distribution, the anatomical localization of D1 sites and the distribution of DA terminals are highly correlated between human and rodent.(Cortes, et al., 1989) The distribution of D2 receptors in human brain also resembles the distribution in the rat and monkey brain.(Kohler & Radesater, 1986; Palacios & A., 1987) Relatively high density of D2 receptors for human and rat brain are found in the SNpc but the SNpr has low density. In human and monkey brain, very low D2 receptor densities are localized while high levels of D2 binding are detected in DA structures of rat brain.(van Domburg & ten Donkelaar, 1991) Based on the similarity in the distribution of D1 and D2 receptors among species, the DA projections from the VTA and the SN in human are assumed to be in good agreement with primate and rodent.(Cortes, et al., 1989; Dawson, et al., 1988; Palacios & A., 1987; van Domburg & ten Donkelaar, 1991) The projections of the VTA and the SN in human brain are similar to the projections in rodent and primate. The common DA efferent connections from the VTA and SN innervate the caudate, the NAc, the olfactory tubercle, the amygdala, and various cortices cross species such as human, primate, and rodent although the primate and human have more DA projections to cortices than rodent have.(S. N. Haber & Fudge, 1997; Paxinos, 1995; van Domburg & ten Donkelaar, 1991) These similarities of the neural connection with the SN and the VTA cross species support that the information from animal

studies can translate into the human model.

In summary, cytoarchitectonic organization of the VTA and the SN is similar between human and other species, such as the rat and the non-human primate. Especially the VTA has more similar organization to the rat than to the non-human primate. These similarities of neural organization provide possibility for functional similarity in these structures. The next section is about the functional similarities of the SN and the VTA between human and animal models.

3. Functional similarity

Lesion in DA neurons of the SN/VTA in both human and animal models demonstrates similar clinical symptoms such as bradykinesia, tremor, memory deficits, and learning difficulties. Neurotoxin and MPTP are commonly used to produce neurodegeneration in the DA nigrostriatal system in rodents and primate.(Arai, et al., 1990; Chang, et al., 1993; Melega, et al., 1996; Sedelis, Schwarting, & Huston, 2001) The MPTP model is similar to human PD because of similar behavioral symptoms and patterns of cell loss.(German, et al., 1996; Heikkila, Hess, & Duvoisin, 1984; J. W. Langston, Ballard, Tetrud, & Irwin, 1983; Manning-Bog & Langston, 2007; Muthane, et al., 1994; Schwarting, Sedelis, Hofele, Auburger, & Huston, 1999; Sonsalla & Heikkila, 1986; Sundstrom, Fredriksson, & Archer, 1990; Sundstrom, Luthman, Goldstein, & Jonsson, 1988; Sundstrom, Stromberg, Tsutsumi, Olson, & Jonsson, 1987) The various animal PD models are showed typical motor

symptoms of PD, such as bradykinesia, tremor, rigidity, gait/posture abnormality, and abnormal coordination, as well as typical cognitive symptoms including memory and learning problem but most motor features of PD are observed in the primate model by a simply identifiable way.(Da Cunha, et al., 2002; Jenner, 2003a, 2003b; Manning-Bog & Langston, 2007; Sedelis, et al., 2001) In addition, DA substitution therapy, a classic pharmacological approach in PD, can treat symptoms in the animal model same as in human PD patient and the long term effect of L-dopa, dyskinesia, are reported in MPTP treated animal model and in PD patient.(Andringa, Lubbers, Drukarch, Stoof, & Cools, 1999; Fredriksson, Danysz, Quack, & Archer, 2001; Gevaerd, et al., 2001; Kuoppamaki, et al., 2007; J.W. Langston, Irwin, Langston, DeLaynnay, & Ricaurte, 1986; Samadi, et al., 2008) The similar treatment works in both animal and human PD model, which means that animal model can be applied in human model for the SN and the VTA.

4. Summary of possibility for human translation

The animal model of the VTA and the SN especially DA system can be translated into human model because of the similarity in chemoarchitecture and cytoarchitecture. The same subdivisions of SN and VTA are recognized and the distributions of D1/D1 receptors are in good agreement cross species. Interestingly, rat is more similar to human in the distribution of VTA subdivisions than primate is. Furthermore, animal models, with loss of DA neuron in VTA and SN, demonstrated similar motor and behavioral symptoms to human

PD patients. These previous studies strongly support the human translation from the animal model in VTA and SN, though not every aspect is matched cross the species.

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Appendix B: Comprehensive Literature Review (II)

WHAT IS THE POTENTIAL OF ANIMAL MODELS TO INFORM OCCUPATIONAL
THERAPY THEORIES AND TREATMENT?

Ji-Hyuk Park

Introduction

Many studies, including human and animal models, contribute to develop OT theories and practice. Many therapists and researchers in OT are more interested in human studies than animal models because a lot of human outcome studies evaluate the effectiveness of OT in practice, and give direct feedback to practitioners about their treatment. Therefore, they can apply the protocol from human research into their practice. In contrast, animal studies cannot be applied directly into OT intervention protocol. However, animal models still provide essential evidences and knowledge to improve OT practice and to develop OT theories as well as human study do.

<i>AREAS OF OCCUPATION</i>	<i>CLIENT FACTORS</i>	<i>PERFORMAN CE SKILLS</i>	<i>PERFORMAN CE PATTERNS</i>	<i>CONTEXT AND ENVIRONMENT</i>	<i>ACTIVITY DEMANS</i>
Activities of Daily Living(ADL)	Values, Beliefs, and Spirituality	Sensory Perceptual Skills	Habits Routines Roles Rituals	Cultural Personal Physical Social	Objects Used and Their Properties
Instrumental Activities of Daily Living(IADL)	Body Functions Body Structures	Motor and Praxis Skills Emotional Regulation		Temporal Virtual	Space Demands Social Demands
Rest and Sleep		Skills			Sequencing
Education		Cognitive Skills			and Timing
Work		Communication			Required
Play		and Social			Actions
Leisure		Skills			Required Body
Social Participation					Function Required Body structures

Figure 1. Aspect of Occupational Therapy's Domain. This figure represents the domain of occupational therapy and is included to allow readers to visualize the entire domain with all of its various aspects. No aspect is intended to be perceived as more important than another. (modified AOTA(Roley, et al., 2008))

Occupational therapy (OT) is a profession concerned with promoting health and well being through occupation. This is defined as “everything people do to occupy themselves, including looking after themselves...enjoying life...and contributing to the social and economic fabric of their communities...”.(Law, Polatajko, Baptiste, & Townsend, 1997; Occupational Therapy Practice Framework: domain and process," 2002) In OT, occupation is not only the final goal (occupation-as-end) but also the way (occupation-as-means) to improve well being.(Kielhofner, 1997; Occupational Therapy Practice Framework: domain and process," 2002; Radomski & Latham, 2008) For example, Occupational therapists can use cooking activities as a therapeutic mean, and also cooking is one of the very important goals for activities of daily living(ADL). OT supports the participation in life by assisting engagement in occupation. The process of OT practice consists of three parts: evaluation, intervention, and outcomes. During the whole process, occupational therapists concern basically five domains: performance in areas of occupation, performance skills, performance patterns, context, activity domains, and client factors (Table 1).("Occupational Therapy Practice Framework: domain and process," 2002) These domains cover various contexts as well as human factors because an essential property of participation in life is the compound interaction between an individual and their contexts (Feature 1).("Occupational Therapy Practice Framework: domain and process," 2002) Based on these domains, OT practice is biopsychosocial and carried out through specific conceptual practice

models(CPM).(Kielhofner, 1997)

CPMs, unique to OT theory, provide rationale and guideline for OT practice. The three concentric layers of knowledge for the foundation of OT theory consist of paradigm, CPMs, and related knowledge(Kielhofner, 1997). The paradigm, a common vision of members in OT, is the inmost core because it addresses the view and the identity of OT in the therapeutic environment. Related knowledge, including concepts, facts, and techniques from other disciplines, complements unique knowledge and applies to OT practice. These three components are not independent of each other and they have mutual influences. CPMs give influence to the paradigm and take influence from both related knowledge and paradigm, called dynamics of the conceptual foundation. The dynamics of this system structure, CPMs more rapidly change than paradigm, which is relatively stable, because a lot of feedback from research, outcome of practice, and CPM application in practice results in steady adjustment of CPMs.

Research, in both human and animal models, generates the new related knowledge to alternate CPMs. During this process, related knowledge from research provides feedback to enrich the related knowledge itself and to make CPMs more rational and effective in practice.(Kielhofner, 1997) For example, the study on constraint-induced movement therapy(CIMT) in stroke patients provided evidence that new modified CIMT protocol improved functions of affected upper extremity. Therapists can use this new protocol directly

in their therapy session. In addition to the evidence in humans, animal studies provide different kind of the related knowledge to support CIMT protocol such as neurological changes after stroke, biological effects of CIMT, and functional improvement. Even though the therapists cannot use exactly same CIMT protocol of monkey research in treatment, interestingly the CIMT concept comes from non-human primate study.

Many occupational therapists and researchers are more interested in human studies than animal studies obviously because occupational therapists treat people with difficulties to participation in occupation and the knowledge from human studies can be applied directly in their therapy. Besides the human studies, animal studies provide different kinds of evidence to influence theory and practice in OT. There are many way for animal model to contribute to OT. The purpose of this scholarly paper is to explore the potential of animal models to informing OT theory and practice especially as it related to neuroscience.

What are the strong points of animal studies?

Before addressing how research with animal model influences occupational therapy, this section is finding out what are general benefits of animal studies to generate essential evidence or related knowledge influencing OT theory and practice. In OT paradigm, humans fulfill their own particular needs or motivation through occupation defined as everything people occupy themselves. Humans have the needs and motivations to participation in tasks

and activities meaningful for themselves, which is referred to as occupational nature or needs.(Kielhofner, 1997) These occupational needs and motivations have biological and psychological foundation. Biologically-based needs may, for instance, be thought of as responses to stages of development in the nervous system, to maintain cardiorespiratory fitness, and to generate motor skills.(Bruner, 1973; Parent, 1978; Rogers, 1983) Psychologically-based needs include the requirement that an individual recognizes a degree of ability and engagement in occupations.(Fondiller, Rosage, & Neuhas, 1990; Reilly, 1962; Rogers, 1983; Smith, 1974; Yerxa, 1967) The individual is satisfied both needs by participating in their own occupation with their own functions. Participation in occupation has effects on biological and psychological status;(Kielhofner, 1997) while the biological and psychological capacity, such as motor and cognitive function, also affects participation in occupation.(Kielhofner, 1997; Law, et al., 1997; Occupational Therapy Practice Framework: domain and process," 2002) Thus, the capacity and occupational performance are interdependent and related to the functioning of the nervous system since these functions stem from core neurological processes.(Lohman & Royeen, 2002; Padilla & Peyton, 1997) This mutual relationship is the theoretical foundation of OT in neurological disease. Animal studies have two advantages to show neurological changes in investigating this reciprocal relationship between occupational participation and recovery in the neural system.

First, investigators can provide same environmental and biological conditions in

which to study animals, something that is impossible in humans. Many factors, such as diet(S. O. Ahmad, Park, Radel, & Levant, 2008), environmental condition(Llorens-Martin, et al., 2007), genetics, age(A. Ahmad & Spear, 1993), and disease, could be outside factors that affect neural changes in neurological studies. In human studies, it is not easy to control these factors because each subject has different characteristics in life style and controlling every factor in humans could be ethical issue. The variation of these factors can be contaminants on an experiment in humans. However, in animal studies, the investigator can more easily control these factors by providing same artificial environment to the target group which has same biological condition. For example, investigators can provide the same cloned condition by using transgenic animals, and providing the same artificial environment by controlling temperature, light, and space. Therefore, well controlled variables, in animal studies, improve the causation of neurological changes because contamination is reduces by controlling variables in the experiment. In addition to controlling external factors, animal models have another methodological advantage to demonstrate precise neurological data.

Second, investigators can use various direct treatments to reveal neurological changes in animal models. There are several methods to investigate biological changes such as electrophysiological, physicochemical, imaging and stereological techniques. Except for some imaging techniques including magnetic resonance imaging(MRI) and computed tomography(CT), most of these are invasive. It is impossible to use such methods in humans

because of practical and ethical issues.(Cramer & Riley, 2008) Hence, investigators have more methodological options to identify neurological changes in animal models than in humans. There are several invasive techniques to measure neurobiological changes. For instance, defining functional areas in the motor cortex is using electrophysiological technique. The microstimulation within the vicinity of the corticospinal neurons under certain anesthetics induce muscle contractions.(Nudo, 2006b) Classically, the functional representation of a given motor cortical area is defined by the muscles contraction initiated by the lowest stimulates current level.(Stoney, Thompson, & Asanuma, 1968) This invasive electrophysiological technique is used to investigate the change of functional representation in cortex after the intervention. Also, the activity change due to treatment in specific deep brain nucleus, such as the caudate-putamen, can be detected by electrophysiological recording procedure.(Glynn & Ahmad, 2003) In addition, stereological methods are very useful to find anatomical changes such as the cell number and the cell body volume of neurons.(Mouton, Gokhale, Ward, & West, 2002; Schumann & Amaral, 2005; West & Gundersen, 1990) By a using computerized stereological system, investigators can count the neurons and measure the cell volume under the microscope. Investigators are limited to use invasive method to prove these types of biological changes in humans. They cannot use invasive techniques, such as stereological methods, in humans because they cannot take the brain until the subject die. Even though researchers may collect anatomical data after the

death, the causation of anatomical changes is less clear because the duration between intervention and data collection is quite long. Animal models can make up for these limitations of human studies. Thus, animal studies provide a more precise picture of neurological changes directly by invasive techniques.

In summary, the nervous system and participation in occupation are interdependent in the OT aspect because neural processes, which are biological psychological function, are essential to participate in occupation and also participation in occupation improves neural process. Thus, it is important to demonstrate the alternation in the nervous system to support this mutual relationship. Animal studies have mainly two advantages over human studies in the investigation of the neurological changes. First, well controlled factors in the experiment reduce contamination in causation of neurological changes. Second, many mythological options, including invasive techniques, increase precision of neurological data. These two advantages are core reasons to make research with animal models more suitable to investigate effects of occupational participation on the neural system. Based on these benefits, many animal studies have demonstrated the positive effect of occupation on the nervous system. The following section is going to be about what and how animal studies contribute to illustrate the constructive neurological changes due to participation in occupation.

Animal studies support positive effects of occupation on the neural system

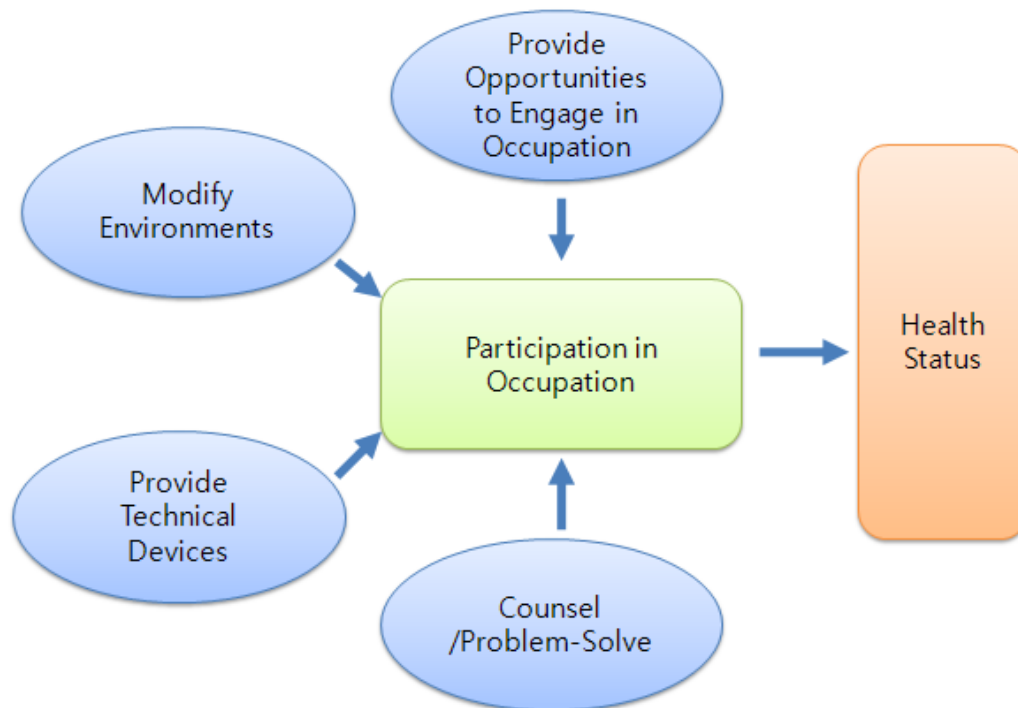


Figure 2. The pathways to employ occupation as therapy

As the previous section mentions, the animal studies is one of the greatest methods to demonstrate the therapeutic use of occupation in the nervous system. This section is going to be about what and how animal studies contribute to develop fundamental theory and practice in OT. In specific CPMs, such as the cognitive-perceptual model, the motor control model, and the sensory integration model, disorder means deficits of neural processing. Therapists use occupation as a means of treatment to improve neural processing, especially in people with neurological disease. There are five main pathways to employ occupation as therapy(Figure 2).(Kielhofner, 1997) Occupational therapists believe that they improve the neurological condition through these pathways especially by providing opportunities to

engage in occupation and modifying environment. This belief, one of core concept in OT practice and theory, is supported by neuroplasticity and neuro-occupation. Neuroplasticity is the capability of neural system to reorganize itself as a result of experience.(Nudo, 2006b; Shaw, Lanus, & van den Doel, 1994) Neuro-occupation, one of OT theoretical construct, is the conceptualization that human occupation and the neural system are interdependent, interactive, and affected by environment.(Lohman & Royeen, 2002) Many animal studies based on these two major conceptual constructs demonstrate this mutual relationship by revealing that characteristics of engagement in occupation, such as required action (motor or physical requirement) and required performance skills, and environmental changes induce to alternate the neurological status.

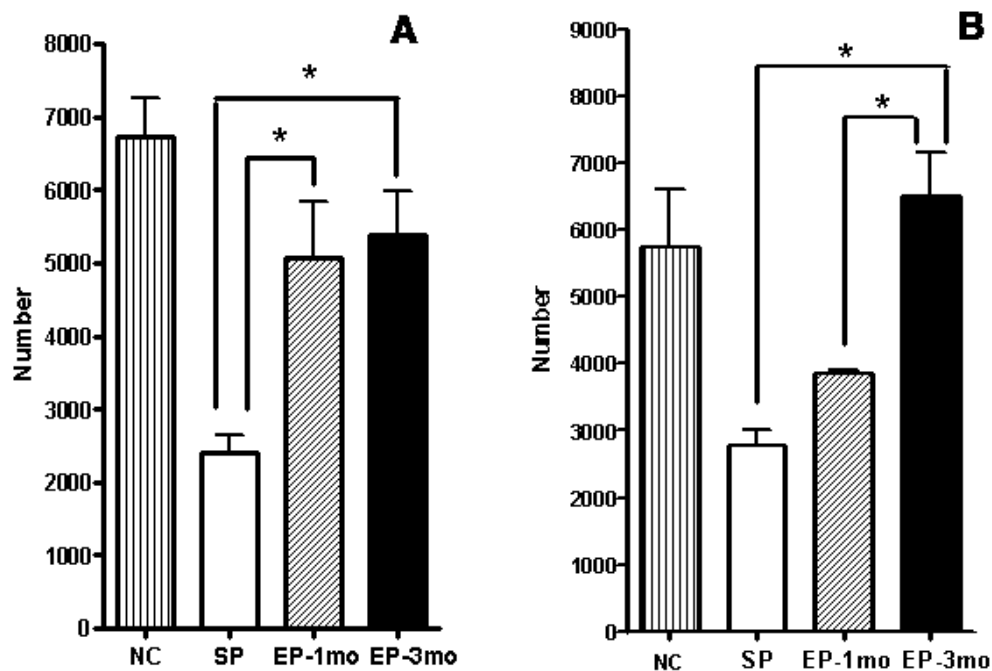


Figure 3. Number of neurons in SNpc(A) and VTA(B) in sedentary and exercised rat Parkinson model.

NC; normal control, SP; sedentary Parkinson, EP; exercise Parkinson.

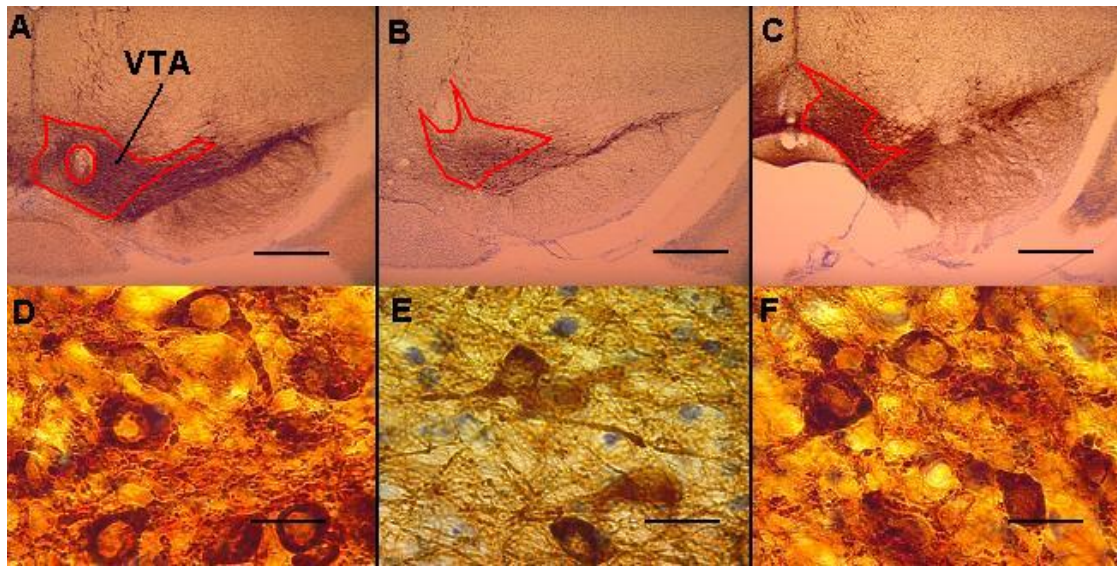


Figure 4. Morphology of VTA neurons in normal control(A,D), sedentary rat Parkinson model(B,E) and exercised rat Parkinson model(C,F).

Studies with animal models prove physical activity improves the function of the neural system. Occupation means doing something meaningful to the individual in OT.(Kielhofner, 1997; Law, et al., 1997) Required action is one of the most important domains in OT, which means engagement in occupation has feature of physical action. Therefore, physical activity is one of occupation used in OT practice for neurological disease. In previous studies, endurance exercise increased the number of neurons in specific brain areas(Figure 3). The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine(MPTP) chronic Parkinson mice were exercised for 40min per a day and 5 days per a week. The speed of motorized rodent treadmill was up to 15 m/min. We provided same diet and same environment to all 4 groups, such as control group, sedentary Parkinson group, 10 weeks exercised Parkinson group, and 18 weeks exercised Parkinson group. We used stereological method with the

computerized system to count the number of dopaminergic(DA) neurons in the substantia nigra pars compacta(SNpc) and the ventral tegmental area(VTA). In both areas, the number of DA neurons was significantly greater in the exercised group than in the sedentary group(Figure 3). Also DA neuron morphology in the exercised group is better than in sedentary group(Figure 4). These results demonstrated that endurance exercise is neuroprotective to DA neurons in the SNpc and the VTA of chronic MPTP mouse model of PD. Research in animal models supports physical activities effect on the nervous system.

Researches in animal models confirm that neuroplasticity is due to skilled demand activity rather than simple repetitive activity. Occupation therapists provide occupation, such as purposeful and skilled activities, to treat people with neurological disorder because engagement improves the nervous system. Various animal studies support experiences of purposeful and skilled activities provide functional improvement. Rats trained skilled motor task demonstrated more neurons and synapses than control the motor cortex.(Kleim, Lussnig, Schwarz, Comery, & Greenough, 1996) Similarly rats, trained in skilled reaching, had more extensive dendritic arborization of motor cortex than non-trained group.(Greenough, Larson, & Withers, 1985; Withers & Greenough, 1989) Electrophysiological mapping showed that reorganization of motor representations in rats trained skilled reaching. The wrist and digit representation increased and elbow/shoulder representation decreased in rats trained skilled reaching in comparison with rats rained unskilled reaching.(Kleim, Barbay, & Nudo,

1998) The same kinds of results have been exhibited in primate studies. Motor representative map stayed stable after performing non-skilled reaching activity (E. J. Plautz, Milliken, & Nudo, 2000) but motor learning changed M1 representational map in a squirrel monkey after having experiences with motor skills. (Nudo, Milliken, Jenkins, & Merzenich, 1996) These animal studies strongly maintain evidence supporting experience-dependent plasticity or learning-dependent plasticity. (Greenough, et al., 1985; Kleim, et al., 1998; Kleim, et al., 1996; Maravall, Koh, Lindquist, & Svoboda, 2004; Rema, Armstrong-James, & Ebner, 2003; Shepherd, Pologruto, & Svoboda, 2003; Withers & Greenough, 1989) In OT, engagement in occupation is physical and mental experience and is required for learning and performing skills. (Kielhofner, 1997; Occupational Therapy Practice Framework: domain and process," 2002; Radomski & Latham, 2008) Therefore, the animal studies on experience- or learning-dependent plasticity firmly support participation in occupation may cause plasticity in neural system, which is implicated as positive effects of occupation on the neural system.

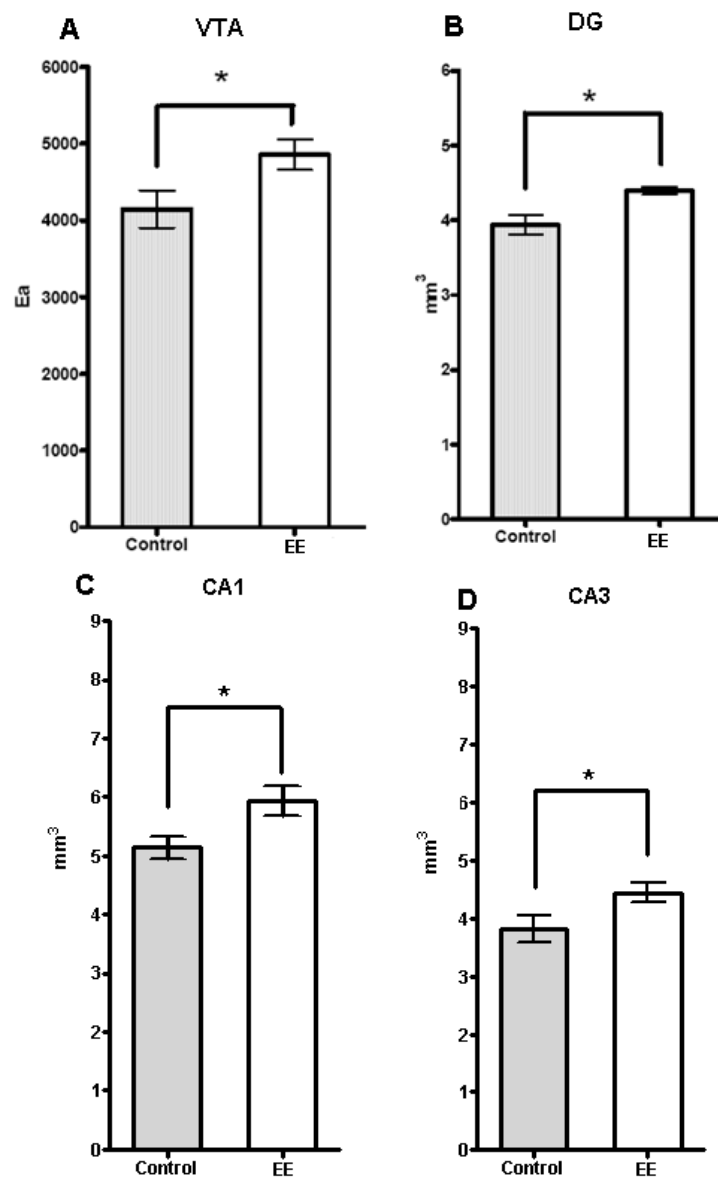


Figure 5. Number of neuron in VTA(A), volume of DG(B), CA1(C), and CA3(D) in normal control and environmental enrichment(EE) rat.

Animal researches demonstrate that environmental changes have positive effects on the nervous system. In animal studies, generally enriched environments(EE) are comprised of bigger housing cages, with objects to interactivity, a running wheel, and tunnels that are changed occasionally to motivate animal exploration.(Bezard, et al., 2003; Laviola, Hannan,

Macri, Solinas, & Jaber, 2008; Rosenzweig & Bennett, 1996) Several studies on effects of EE on neurological disorders, such as PD,(Bezard, et al., 2003) Huntington's disease,(Nithianantharajah, Barkus, Murphy, & Hannan, 2008; Spires, et al., 2004; van Dellen, Blakemore, Deacon, York, & Hannan, 2000) and Alzheimer's disease,(Berardi, Braschi, Capsoni, Cattaneo, & Maffei, 2007; Gortz, et al., 2008; Jankowsky, Xu, Fromholt, Gonzales, & Borchelt, 2003) revealed that EE enhanced experience-dependent plasticity in transcription of specific genes, synaptogenesis, and adult neurogenesis in the rat model.(Laviola, et al., 2008) In our pervious study in rats without neurological disorder, EE increased the number of neurons in the SNpc and the volume of hippocampus specifically in dentate gyrus(DG), CA1, and CA2(Figure 5). Also morphologically the EE group was better than control group. The positive effects of EE on the neural system support modifying environment in OT as a treatment approach for people with neurological deficits.

In addition to the neurological evidence, research in animal models also provides functional data to support positive effects of occupation on the nervous system. These functional or behavioral data are related to the neurological data. Cortical reorganization reduced failures in reaching task in rats.(Kleim, et al., 1998) EE improved functions, such as maze performance, as well as cellular of pathogenesis in AD rat.(Arendash, et al., 2004; Jankowsky, et al., 2005; Jankowsky, et al., 2003; Lazarov, et al., 2005; Levi, Jongen-Relo, Feldon, Roses, & Michaelson, 2003) In Parkinson rats, endurance exercise improved running

ability and motor control as well as the number of neurons in the SNpc and the VTA.(Petzinger, et al., 2007) The positive behavioral improvement due to experience-dependent plasticity allows them engage in occupation such as feeding, locomotion, and navigation. The coupling between neurobiological and behavioral improvement due to experience-dependent plasticity in animal models is solid evidence for fundamental OT theory.

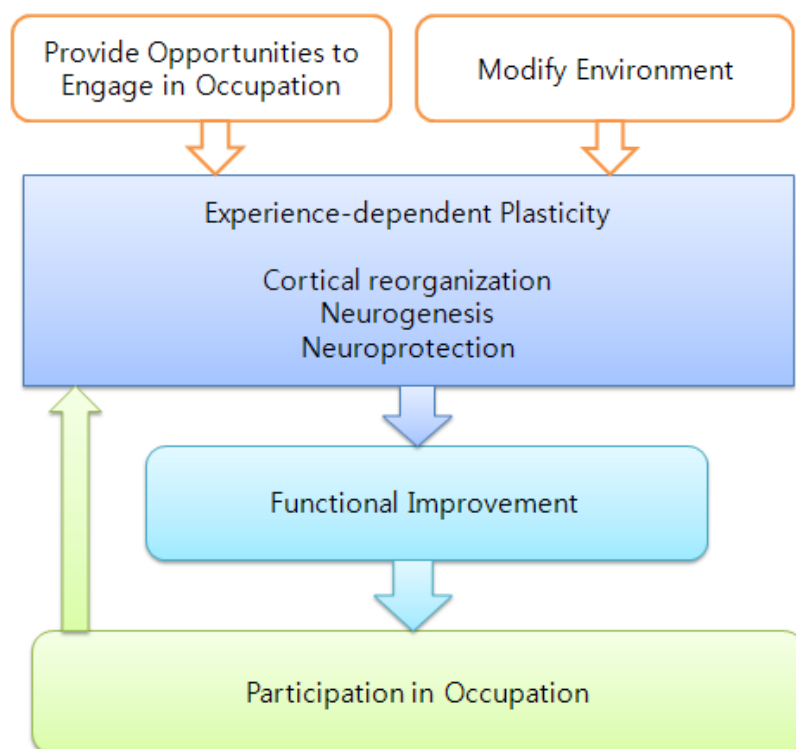


Figure 6. Dynamics of employing occupation as therapy in neurological system.

In summary, animal models prove positive effects of major two methods to employ occupation as therapy, such as engagement in occupation and environmental changes, on the nervous system. The plasticity due to experience driving occupation or environmental

enrichment is the reason of functional improvement. These positive effects on occupation and environment, neurological and functional improvement, are key for OT practice in neurological diseases. Occupational therapists provide the opportunities of engaging in occupation and modify the environment to treat people with neurological disorder. Based on OT theory, the neurological improvement, due to providing of occupational therapy, enhances the functional improvement which allows people participate in occupation. In OT's view, through the participation in occupation, the individual can maintain the health status and quality of life. Animal studies have potential to prove this theoretical link of OT practice by providing the evidence for experience-dependent plasticity due to engaging in occupation or modifying environment. Also animal models demonstrate the link between the behavioral or functional improvement and neurological improvement. The strong support of animal studies contributes to establishing strong foundation of OT theory and practice in neurological disorders. The following section is going to about what kind of potential animal studies have for OT in humans.

Animal studies provide possibilities and evidence of treatment in humans

The previous section confirms that animal studies demonstrate OT approaches can induce the change of neurological status. This section is about what are benefits from animal models for OT in humans. Many kinds of animals, including rats, mice, and monkeys, are

used for various purposes in neuroscience. Some of these studies are to investigate the effect of a specific treatment on particular neurological diseases.(Nichol, et al., 2008; Quik, et al., 2003; Whishaw, Alaverdashvili, & Kolb, 2008) Through these studies with animal models of disease, the researchers provide the possibility for human translational research.(Miltner, Bauder, Sommer, Dettmers, & Taub, 1999; Schneider, et al., 2007; E. Taub & Uswatte, 2003) Based on the knowledge from animal studies, researchers design human outcome studies and may expect similar result in humans. For example, endurance exercise preserves the number of DA neurons in the VTA and the SNpc of PD rats. Researchers hypothesize that endurance exercise would preserve the number of DA neurons in people with PD that would attenuate symptom. In this case, the previous study in rat PD model provided possibility of endurance exercise as therapeutic intervention in human PD. Without rat PD model, this kind of translation study in humans is impossible. The animal disease model is essential in human translation study. There are some benefits from animal model for translation research into humans.

Researchers can effectively test their treatments and hypothesis on mechanism of neurological disease in animal models. Animal models of neurological disease have been used to inform the mechanism of human disease.(Chen, Hsu, Hogan, Maricq, & Balentine, 1986; Ling, Lee, & Kalehua, 2004; Nehls, Cartwright, & Spetzler, 1986; Smeyne & Jackson-Lewis, 2005) Neurological deficits in animal models are induced by an artificial method

based on the mechanism of human disease and neural network. Animal studies use large samples to contribute to build a specific disease model. Based on accumulated knowledge from these studies, researchers developed the animal model of a neurological disease similar to human disease. For example, neurotoxin and MPTP are commonly used to induce parkinsonism in rodent and primate because this substance induces neurodegeneration in the DA nigrostriatal system.(Jenner, 2003a, 2003b; Smeyne & Jackson-Lewis, 2005) The similarity motor disturbances and neural circuit changes between animal PD model and human PD supports that MPTP animal model is the closest to human PD.(Burns, et al., 1983; Manning-Bog & Langston, 2007; Wichmann & DeLong, 2003) Thus, researchers can test their treatment protocols, including pharmacological and non-pharmacological, in animal MPTP model before applying it to human. They evaluate the effect of their protocol by behavioral and neurological changes in the animals. Huge number of studies in MPTP animal model contributes to development neuroprotective therapies in PD and activity of antiparkinsonian drugs in humans can be highly predicted based on response of non-human primate MPTP model.(Jenner, 2003a, 2003b; Manning-Bog & Langston, 2007; Wichmann & DeLong, 2003) In addition, the animal model of a neurological disease help OT researchers and practitioner to understand the mechanism and neural network related to the disease. For instance, various animal studies contributed to build the model of neural network of the basal ganglia linked to PD.(Aldridge, Berridge, & Rosen, 2004; Graybiel, 2001; Tamminga &

Holcomb, 2001; Yasoshima, et al., 2005) Through studies in animal disease models, OT researchers and practitioner update their knowledge on mechanism and the neural network related to the specific neurological disease. With this knowledge, OT researchers and practitioners understand new treatment approaches including both OT and non-OT treatment. Also animal models for neurological disease build the basic foundation for transitional research in humans.

Studies in animal disease models provide basic evidence to initiate studies in humans with neurological disease. The one of general purposes in human studies on neurological disease is to investigate the effect of treatment. Before applying treatments in humans, researchers should show the enough evidence to explain how the treatment works on the disease and what effects are expected. Accumulated studies in an animal model of the neurological disease provide evidence of neurological changes due to the intervention in the target area. The constraint-induced movement therapy(CIMT), one of the most interesting treatment approaches in OT, was derived from early primate studies.(Miltner, et al., 1999; E. Taub & Uswatte, 2003) The core concept of CIMT, learned nonuse, came from early studies with monkeys in whom somatic sensation was eliminated surgically from unilateral upper extremity by dorsal rhizotomy. The monkeys regained use of affected extremity by forced training affected upper extremity or restricting the movement of unaffected upper extremity.(E Taub, 1980; E. Taub, Heitmann, & Barro, 1977) Based on this primate study, the

same mechanism was thought to apply to human with motor deficits of upper extremity due to neurological disorder such as stroke and cerebral palsy.(Blanton, Wilsey, & Wolf, 2008; Boake, et al., 2007; Bonnier, Eliasson, & Krumlinde-Sundholm, 2006; Charles, Wolf, Schneider, & Gordon, 2006; Gordon, Charles, & Wolf, 2006; B. Hoare, Imms, Carey, & Wasiak, 2007; B. J. Hoare, Wasiak, Imms, & Carey, 2007; Kwakkel, Rietberg, & van Wegen, 2007; Mennemeyer, Taub, Uswatte, & Pearson, 2006; Sutcliffe, Gaetz, Logan, Cheyne, & Fehlings, 2007; E. Taub, et al., 2006) Occupational therapists use CIMT with people with motor deficits of the upper extremity to improve the level of physical function and participation in occupation of daily living.(Gillot, Holder-Walls, Kurtz, & Varley, 2003; Martin, Burtner, Poole, & Phillips, 2008; Page, Levine, & Hill, 2007) The early monkey study provided basic evidence to initiate applying CIMT in humans. Thus, studies in animal models have potential to develop new treatment approaches in humans with neurological disease.

Research in the animal model of a neurological disorder helps develop more effective intervention in humans. After neurological damage or pathological onset, the nervous system starts to change in various ways.(Nudo, 1999, 2006a; Nudo, et al., 2003; E. Plautz & Nudo, 2005) Animal studies have provided evidence to understand of these processes, including recovery process, in the neural system. A better understanding of neurological changes after a neural incident is helpful to develop therapeutic approaches in humans based on

pharmacologic, cell-based, gene transfer, immune-based, and occupational therapeutic knowledge.(Cramer & Riley, 2008; Heddings, Friel, Plautz, Barbay, & Nudo, 2000) Evidence has contributed to implement and design clinical trials to investigate the effects of treatment for neurological disease. Generally direct measurement of biological events, including cellular and molecular events, is not possible in humans.(Cramer & Riley, 2008) In addition, researchers can investigate to find the important factors that improve effectiveness of interventions in animal models with controlling pathological degrees, biological factors and environmental factors. In the rat model of unilateral ischemic stroke, investigators compared motor skill training and voluntary exercises to find which training factor would enhance recovery after stroke.(Maldonado, Allred, Felthouser, & Jones, 2008) In the primate model in which unilateral forelimb is surgically deafferent, researchers investigated minimal period of restricting movement of the intact upper extremity. When restricted after surgery, motor recovery improves in the deafferent limb.(E. Taub & Uswatte, 2003) These animal studies contribute to human protocols. Thus, animal models have potential to improve the OT approaches for neurological diseases.

In summary, animal models of neurological diseases are essential to improve OT practice and theory. The animal models provide related knowledge for a better understand the mechanism of diseases and related neural networks. Based on this knowledge, researchers can test their hypothesis of neural disease. In addition, accumulated animal studies in

neurological disease contribute to introduce the new approaches to human diseases and to improve the effectiveness of treatment. Therefore, animal models of neurological disease have the potential to improve OT theory and practice by providing evidence and a fundamental base for human transitional study in neurological disease.

Conclusion

The purpose of OT is to improve health and well-being in humans through engagement in occupations. However, it does not mean only human studies improve OT theory and practice. Animal models have contributed to improve OT theory and practice especially in neurological disease. Based on precise neurobiological data derived from experimental and methodical benefits, animal models support core OT theory which demonstrated positive effects of occupation and environment on the nervous system. Evidence from animal studies influences not only OT theory but also the practice approaches. Animal models improve CPMs by enriching enriched the related knowledge especially with sound neuroscience and by providing fundamental structures for human transitional studies in neurological disease. Animal models for neurological diseases provide basic knowledge and evidence of neurological disease, contribute to modify the interventions to be more effective in humans, and provide evidence to design well built human studies. However, there were very few animal studies in OT area.(Lane, 1998; Roughton, Schneider, Bromley, & Coe, 1998; Wood, 1996) Recently OT researchers have become interested in animal models for neurological disorders. They invented the Sensory Processing Scale for Monkey(SPS-M) to develop the nonhuman primate model of sensory processing disorder.(Schneider, et al., 2007) This kind of work contributes to build up OT approaches for SPD and provides related knowledge on SPD to OT researchers and practitioners. In conclusion, animal models of

neurological disease are critical and have the potential to improve OT practice and theory in many ways. Therefore, OT researchers need to pay more attention to animal models as well as human studies.

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Appendix C: Comprehensive Literature Review (III)

HOW DOES STEREOLOGY HELP TO INFORM TRANSLATION FROM
NEUROSCIENCE TO OCCUPATIONAL THERAPY?

Ji-Hyuk Park

Introduction

Occupational therapy(OT) is a health profession that helps an individual to improve well-being and quality of life (QOL) through participation in occupation.(Kielhofner, 2004; Russ & Robert, 2000) One of the important domains in OT is performance skills which include sensory perceptual skills, motor and praxis skills, emotional regulation skills, cognitive skills, and communication/social skills. An individual needs these skills to participate in everyday occupations.(Roley, et al., 2008) All of these skills are supported by integrated neurological processes. An individual with neurological dysfunction therefore may have difficulties in participating in some or all occupations. Occupational therapists help individuals with neurological disorder to participate in occupation, by using specific OT interventions to improve neurological status due to experience-dependent neuroplasticity. Stereology can analyze biological tissue in anatomical and cytoarchitectural features such as the number of neurons, the volume of neurons, and the volume of specific areas in the brain (Peter R. Mouton, 2002) Morphological changes in neurons and other cell population in brain are related to brain functions.(Bright, Moss, Stamatakis, & Tyler, 2008; Edwards, Liu, & Blumhardt, 2001; Sajdel-Sulkowska, Nguon, Sulkowski, Rosen, & Baxter, 2005) These biological changes are based on experience-dependent structural plasticity and structure-function relationship in neuroscience. Stereology robust tool when employed to investigate

morphological changes in neurons, cortex area, and specific parts of brain involved in special brain function.(Bussiere, et al., 2003; Nowak, Nussdorfer, Nowak, Mazzocchi, & Malendowicz, 1990; Schmitt, et al., 2009; Scott, Diaz, & Ahmad, 2007; Stachowiak & Malendowicz, 1993) This paper will explore how stereology can be used to inform translation of neuroscience knowledge to OT practice.

What is stereology?

Stereology is an interdisciplinary field focused on analyzing biological tissue with the three-dimensional interpretation of planer sections using estimating methods and mathematically unbiased sampling.(Peter R. Mouton, 2002) The primary parameters measured in stereology are volume, number, length, and surface area. The approach has been developed based on geometric principles, which Egyptian architects discovered and Greek builders and mathematicians refined. For the Greeks, Euclid was the major contributor to build up geometry as a tool for understanding mathematical relationships and constructing structures and objects. However, biologists cannot apply this classical geometry in biological structures because biological objects have a varying and arbitrary nature and are also quite variation between and within the groups. Despite the classical geometry's limitation in application to biological objects, biologists have been using assumption- and model-based approaches based on classical geometry long before unbiased stereology was

developed.(Peter R. Mouton, 2002)

A number of contributions developed the theoretical foundation for unbiased stereology to make this approach an efficient and accurate estimate of first-order stereological parameters, (number, volume, length and surface area). In 1635, Cavalieri created a method to estimate the mean volume of non-classically shaped object.(H. J. Gundersen, 1986; H. J. Gundersen & Jensen, 1987; Mayhew & Olsen, 1991; Nurcombe, Wreford, & Bertram, 2001) This manner allows estimating the total volume of any biological object from the sum of sections selected by systematic-random sampling through objects. Cavalieri method is theoretically unbiased, which means it avoids systematic error. The French naturalist Buffon made another important theoretical basis for modern stereology in 1777 at the Royal Academy of Sciences in Paris. Buffon used needle problems, where a needle is thrown at random on a floor with a grid of lines to demonstrate that, and the chance of the needle intersecting with a line is directly proportional to the length of the needle. Buffon's principle makes it possible to estimate the total length and the total surface area of non-classically objects without further mathematic assumptions.(Buffon, 1777) In 1847, the French geologist and mining engineer Delesse proved that the volume of objects is proportional to the total profile area of each cut surface through objects.(M. A., 1847; Peter R. Mouton, 2002; Russ & Robert, 2000) Contemporary stereologists use Delesse's law to estimate the volume of biological objects from the profile areas of sections in random selection. These theoretical

principles, developed from the fifteenth to the eighteenth centuries, provide the fundamental basis for unbiased stereology. In the twentieth century, based on former theoretical foundations, unbiased stereology is possible. (Peter R. Mouton, 2002; Russ & Robert, 2000)

In 1925, Swedish mathematician Wiksell illustrated the corpuscle problem, which is the difference between the number of objects of cross-sectional area and the number of objects of 3-D volume, to show precise estimates of the number of arbitrary and non-classically shaped biological objects cannot be achieved from profile counts on 2-D histological sections. (Peter R. Mouton, 2002; Russ & Robert, 2000; Wiksell, 1925, 1926) The “corpuscle problem” arose from Wiksell’s desire to know the number of thyroid globules in a thyroid gland. Wiksell found he could not estimate the number of the globules in a given thyroid gland from the number of globules on the cross-sectional surface. (de Gunst & Luebeck, 1998) Since first describing the problem, a couple of attempts have not succeeded to overcome the problem by using formula with correction. In 1984, D. C. Sterio eventually overcame the “corpuscle problem” by his disector method to estimate the actual number of objects in a given tissue without assumptions. This was the first theoretically unbiased method based on probability theory. (Sterio, 1984) The disector method uses a disector pair, two virtual planes, and two principles, an unbiased counting frame and unbiased counting rules, and was presented by Gundersen in the late 1970s. (H. J. G. Gundersen, 1977) Through the disector method, the researcher can avoid severe errors of bias when analyzing 3-D morphological structures from

2-D sections. The disector method is a crucial component of unbiased stereology because it is based on probability theory rather than mathematical assumption. Assumption-based geometric formulas do not adequately estimate the morphology of biological objects which have naturally random variables.(H. J. Gundersen, 1986; Sterio, 1984)

Stereology offers methodological advantages for neuroscience research in particular. Over the past forty years, modern stereology has eliminated several theoretical obstacles to allow precise estimates in the 3-D parameters derived from 2-D cross-sections of biological issues. These theoretical breakthroughs form the methodological foundation of unbiased stereology. The initial step toward modern unbiased stereology was published in 1971.(Elias, 1971) In this article, Elias noted that 2-D image data generate errors in estimating the 3-D parameter of biological objects because biological objects are not of classical shapes, such as a sphere, a cone, and a cylinder. Thus each part of 3-D objects does not have an equal chance to be selected by cross sectioning. These sources of bias do not allow using assumption-based geometry in biological objects, which are one type of systematic error.(West, 1999)

In addition to these methodological advantages, computer microscopy contributes to development of a powerful stereological system.(Glaser & Glaser, 2000) The integration of stereology with computer assisted morphological mapping implements efficient method to make unbiased estimate on many kind of biological tissues. A computerized stereological system improves efficiency and accuracy in estimation. It has a three axis computer-

controlled motorized stage which can rapidly access particular locations and make systematic random sampling throughout the entire tissue specimen regardless of optical magnification. The system can map the interest area without overlap and save all of the acquired data. These mapping and data storage technique make it possible to generate maps easily in any desired orientation, to perform stereological computation analysis, and to carry out reconstructions. Also researchers can access easily previous studies and share their findings and related maps within scientific community.

In summary, stereology is the scientific method to estimate physical parameters of 3-D, objects from 2-D cross-sectional data. This theoretically unbiased technique provides methodological advantages for researchers to investigate morphological features in biological objects, such as neurons,(Grady, Charleston, Maris, Witgen, & Lifshitz, 2003; Nurcombe, et al., 2001; A. M. Smith, Pappalardo, & Chen, 2008; Tang, Lopez, & Baloh, 2001) liver tissue,(Altunkaynak & Altunkaynak, 2007; Santos, et al., 2009) tumors,(Bonnelykke-Behrndtz, Sorensen, & Damsgaard, 2008) and lung(Basoglu, Buyukkarabacak, Sahin, & Kaplan, 2007; Nielsen, et al., 2001; Weibel, Hsia, & Ochs, 2007). In addition, computerized system improves accuracy and efficiency in stereological estimation process. In neuroscience, stereology is used to study changes in anatomical structures as a consequence of neurological disorder, therapeutic treatment, or pathology.

What biases affect the precision of stereological estimates?

In stereology, two main sources of variation, systematic and nonsystematic error, influence the accurate of estimate of a parameter (Figure 1).(Peter R. Mouton, 2002)

Systematic error (variation) is a source of bias that causes inaccurate estimation in stereological parameters. This error is a bias in measurement which makes significant difference between estimation and actual value.(Peter R. Mouton, 2002; Russ & Robert, 2000)

There are two types of systematic error; stereological bias and non-stereological bias (Figure 1). Stereological bias is about estimate method. Stereological method is a measurement tool to estimate stereological parameter (such as cell number or volume) in the population of structures. If this measurement tool is not accurate, the outcome of measurement is not correct. For example, with the ruler stretched because of heat, we cannot measure the length

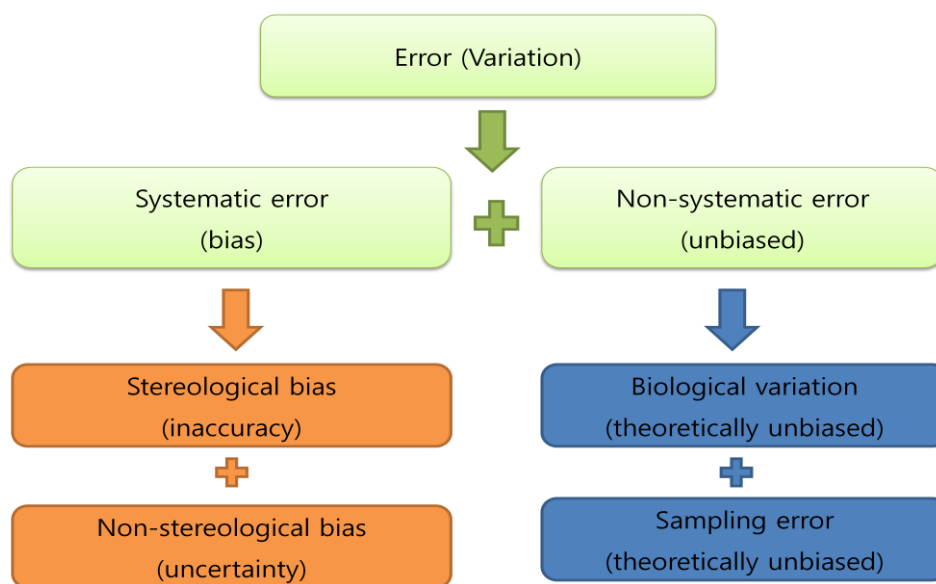


Figure 1. Sources of variation: Total error consists of systemic error and non-systemic error. Systemic error is bias (stereological and non-stereological), which means it can be avoided by investigators. However, nonsystematic error is unbiased and cannot be eliminated by investigators because it is theoretically unbiased, which means it is not related with theoretical weakness.

accurately. Since the true value generally is unknown, researchers require the precise measurement tool to estimate stereological parameters. Like a stretched ruler, the inaccurate or biased estimate method cannot estimate stereological parameters precisely because it generates stereological bias. It is impossible to remove this stereological bias because it is inherent in the methodological theory and process employed. The only way to remove this stereological bias is to change the method. Researchers need unbiased design-based stereology to remove the methodological and stereological bias. The second type of systematic error is non-stereological bias. It includes incomplete staining, ascertainment bias, improper calibration and observation bias. It can, however, be avoided. (Peter R. Mouton, 2002) Also computerized system helps avoid these non-stereological biases. (Glaser & Glaser, 2000) In addition to systematic error, nonsystematic error also affects data but it is not sources of bias.

Non-systematic error, either biological variation or sampling error, also contributes to an error estimates (Figure 1). (Peter R. Mouton, 2002) Due to biological variation, the expected value of a parameter is often different between individuals. Biological variation can be reliably estimated reliably from sample estimation without all bias being known (such as stereological or non-stereological error). (Peter R. Mouton, 2002) The total observed variance (CV^2) is the sum of relative variance (CE^2) and biological variance (BV^2), and we can

estimate CV^2 and CE^2 from sample estimation.(West, 1993a)

$$\text{Total observed variance} = CV^2 = BV^2 + CE^2$$

Therefore, it is possible to assess how much of the BV^2 contributes to the total variation in a sample estimate. Increasing the number of samples can reduce BV^2 but only increasing sample size does not lead to accurate estimate without unbiased method based on probability theory.(West, 1993a) Besides biological variation, sampling error is another source of random variation in the sample estimate.(West, 1993a, 2002) Sampling error or variation is due to observing a sample rather than the whole population. In statistical term, sampling error refers to the coefficient of error (CE). Two factors mainly contribute to the amount of CE in stereology, which are the number of sections (between-section variation) and the number of regions within each section (within-section variation).(West, 1993a) The precision of sample estimates can be decreased by insufficient sampling in sections or within each section. Researchers need the sampling method to minimize sampling error to obtain accurate and precise data.(Mandarim-de-Lacerda, 2003)

In summary, two main sources of error influence the level of accuracy in stereological estimate, systematic and nonsystematic variation (Figure 2). Systematic errors may be due to biased measurement, poor histological processes, or calibration error. Stereological bias is introduced by an assumption of uniformity that typically does not fit biological objects, and this assumption can skew the data even with precise sampling or after

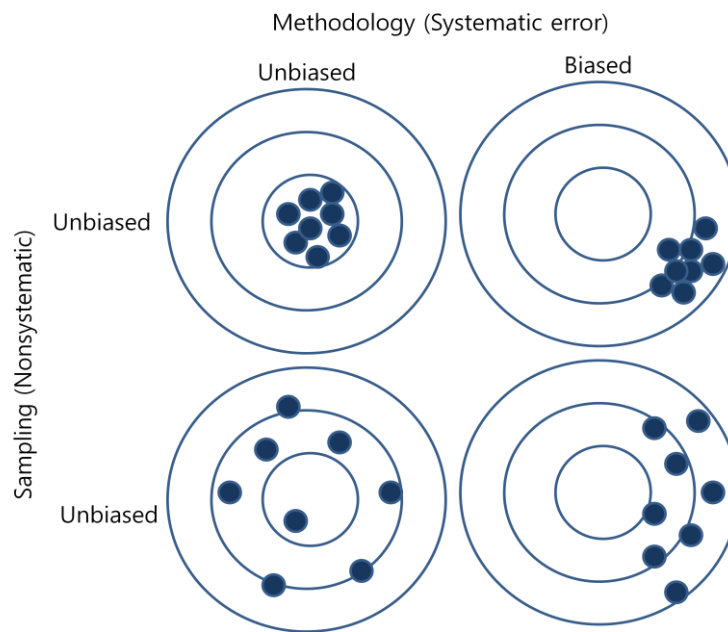


Figure 2. Expected results depending on methodology and sampling method. Number estimates are represented by solid black circles and the true number is represented by the central gray circle. Note that the means of unbiased estimates with both the precise and imprecise sampling are equal to the true number but not the case with biased estimates either with precise or imprecise. Unbiased methodology increase validity and precise sampling increase reliability.

increased sampling. In addition to systematic error, non-systematic variation, biological variation or sampling error, contributes to the total variation inherent in the data. This total variation is due to random error, and described as the mean-squared difference between estimated value and expected value. By contrast to systematic error, nonsystematic error is unbiased, which means it can be measured from the sample estimate. For an accurate estimate of a parameter in biological object of interest, researchers may to minimize both systematic and non-systematic variation through both unbiased methodology and precise sampling.

What are methodological benefits of stereology?

In the 1980s, two breakthroughs overcame main sources of biases, encouraging application of stereological method in the analysis of biological objects. In 1984, Sterio

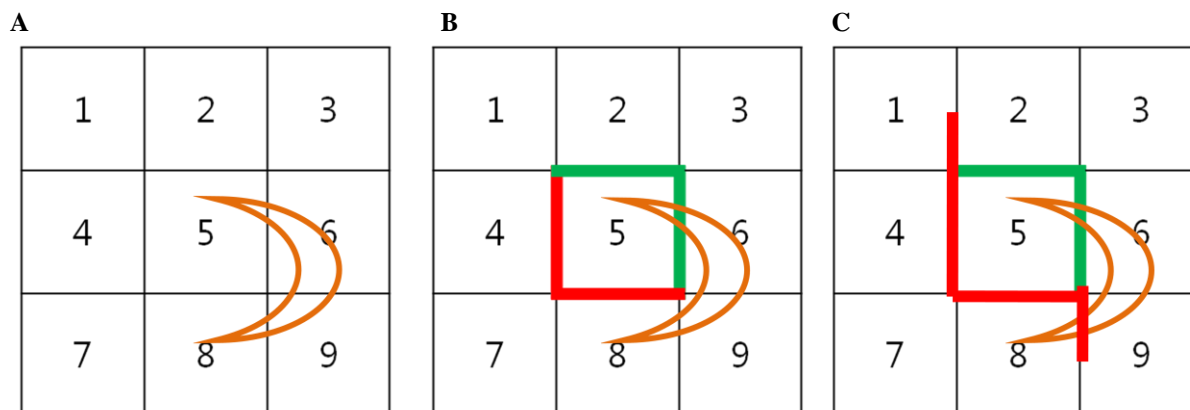


Figure 3. Counting frames. Red line is exclusion line and green line is inclusion line. The object should be counted when it is within the frame or touches the inclusion line (green line) and should not be counted when it is out of the frame and touches the exclusion line (red line). **A.** Counting frame without inclusion and exclusion line; the crescent object can be counted in frames 5, 6, 8, and 9. **B.** Counting frame with biased inclusion and exclusion line; the crescent object can be counted in frames 5 and 8. **C.** Counting frames with unbiased inclusion and exclusion line; the crescent object can be counted only in frame 8.

introduced the disector principle to estimate the number of biological objects in a given volume of tissue.(Sterio, 1984) He employed an unbiased geometric probe in the disector principle with unbiased counting frame and unbiased counting rules. In the unbiased counting frame, the object should be counted when it is within the frame or touches the including line(green line) and should not be counted when it is out of the frame and touches the excluding line (red line) (Figure 3). In this principle and unbiased frame, the chance for the object to be counted in the volume tissue is equal for each object no matter what shape and size of each object may be (Figure 3). Without further assumption of the shape and size, the disector method can be used to estimate the total number of biological objects in a given volume from small sample and to extrapolate that estimate to the expected value at the population level.(H. J. Gundersen, 1986; Sterio, 1984) A second procedural breakthrough was application of systematic-random sampling in biological reference spaces.(H. J. Gundersen

& Jensen, 1987) Systematic-random sampling made it possible for the disector method to be applied efficiently to biological tissues without bias. This sampling method guarantee sufficient within-individual sampling to save effort, time, and resources wasted to minimize biological variation influencing sample estimation.(Peter R. Mouton, 2002) Combining the disector method and the systematic-random sampling, modern stereology is a powerful tool that can be used to obtain precise estimates of morphological parameter efficiently in a biological population.(Peter R. Mouton, 2002; Schmitz & Hof, 2005; West, 1993a, 2002) The topic of this section is methodological advantages of modern stereology.(Mandarim-de-Lacerda, 2003)

Table 1. Sampling hierarchy for biological tissues.

Level	Sampling	Magnification	Example
Individual	Independent-random	Low	Human, monkey, mouse
Tissue	Systematic-random	Low	Brain, heart, lung
Slab/section	Systematic-random	Low	Cortex, vessel, epidermis
Probe	Systematic-random	Low/high	Points, lines, planes, volume

Systematic-random sampling is theoretically unbiased and increases the efficiency of stereological estimate.(H. J. Gundersen, 1986; H. J. Gundersen, Jensen, Kieu, & Nielsen, 1999; West, 1999) Randomness is the most important sampling principle to estimate the mean of a parameter from sample in whole population. Random sampling gives each object same probability to be selected. Without random sampling, each object no longer has an equal probability to be chosen, which introduces sampling bias that distorts the estimation of a

parameter. Therefore, researchers in stereology use a sampling hierarchy for biological tissues (Table 1). While all of the sampling methods are random sampling, only in the level of individual is independent-random sampling used. The remaining levels, such as tissue, slab/section, and probe, use systematic-random sampling because systematic-random sampling is more efficient than independent-random sampling although both of them are theoretically unbiased.

Systematic-random sampling reduces effort and time in estimating a parameter in biological object of reference area relative to independent-random sampling. Systematic-random sampling is a sampling method involving the selection of elements from an ordered sampling frame. In systematic-random sampling, every k^{th} element in the frame is selected. k is the selection interval calculated as:

$$\text{Selection interval (k)} = \text{Population size (N)} / \text{Sample size (n)}$$

$$\text{Sample size (n)} = \text{Population size (N)} / \text{Selection interval (k)}$$

For example, if the cortex of a rat is represented over 18 successive histological sections (N) and 9 separate observations (n) are required, the selection interval is “2” (k) (18 sections / 9 observations = 2). Select nonzero random number between 1 and 2, and then sample every other section because the section interval (k) is 2. If you select the slide 2 for the first sample

slide, the numbers of your sample slides will be 2, 4, 6, 8, 10, 12, 14, 16, and 18; like summarized in the following diagram.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18

A

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18

B

Figure 4. Systematic-random sampling (A) vs independent-random sampling (B). Systematic-random sampling (A) makes only two sets of sample groups from 18 slides when the sample size (n) is 9. The number of population (N) is 18. Selection interval (k) is 2. The One set includes slide 1, 3, 5, 7, 9, 11, 13, 15, and 17. Another set includes slide 2, 4, 6, 8, 10, 12, 14, 16, and 18. However, independent-random sampling (B) has more than two sets of sampling groups.

With systematic-random sampling, the number of sets is just two in this example. Just two times estimating in two set of the sample can obtain the expected value. However, with independent-random sampling, the number of sampling sets could be more than two because repeating estimates decreases the variation of estimate with repeated independent-random sampling (Figure 4). Therefore, researchers can save their time and effort with systematic-random sampling in biological objects. In addition to efficiency, systematic-random sampling equally covers from the beginning to the end of a biological object. The goal of sampling in stereology is to capture the most biological variability within the reference area of interest to minimize the sampling error. However, through independent-random sampling, the sample does not always cover the whole reference area, which increases sampling bias (Figure 4).

For example, the second example sets of independent-random sampling includes slide 3, 9, 11, 12, 13, 14, 16, 17, and 18. If slide 1 is the anterior side of rat brain, this sample set selects more posterior parts of brain. It does not equally cover from the anterior to the posterior of the brain.

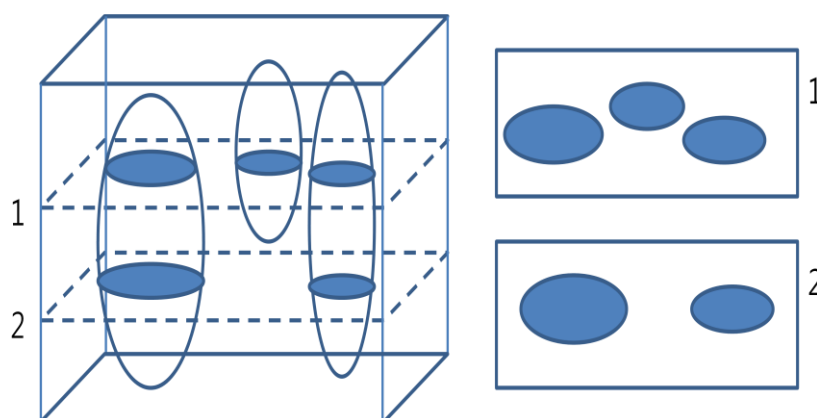


Figure 5. Bias to estimate number in 3-D object from 2-D profiles The corpuscle problem. Three objects with arbitrary size, shape, and orientation appear as five profiles on 2-D sections.

In addition to the sampling method, the disector principle provides methodological advantage to modern stereology. This principle is based on probability theory, and thus overcomes bias in estimating accurate object number in defined biological reference area. (Bendtsen & Nyengaard, 1989; Fiala & Harris, 2001) In 1847, Delesse demonstrated differences between the number of object per unit of volume (N_V) and the number of object per unit of cross-sectional area (N_A) because one 3-D object can appear more than one time in the cross-sectional profile area (Figure 5). (Sterio, 1984) The probability for each object to be selected depends on the shape, the size and the orientation of the object. For example, a large

object, or an object with an irregular shape, can be counted more than once because it appears in several different profile areas. The dissector principle solves this unequal probability problem. The 3-D virtual dissector probe gives every 3-D object of the reference area same probability to be counted, which means one object would be counted just one time. Therefore, with this unbiased 3-D probe, investigators can estimate how many objects are in the reference area no matter the shape, size, and orientation of the object.

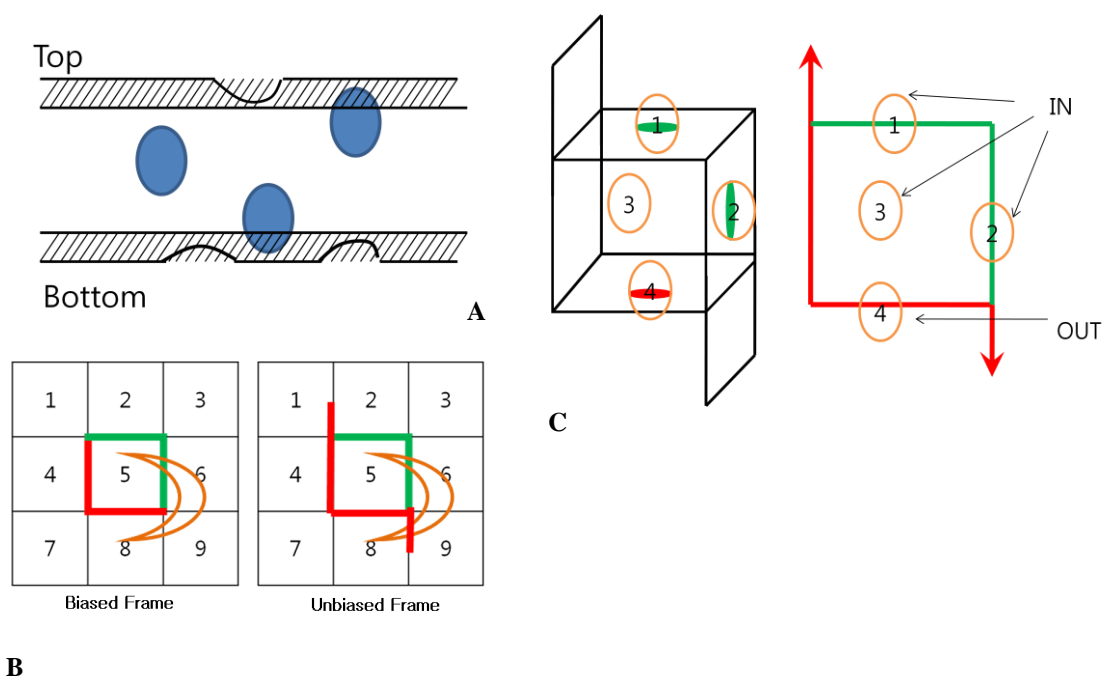


Figure 6. A. Guard volume; Schematic view of an optical dissector frame. Hatched lines indicate guard volume to avoid artifacts at section surface. h , height of dissector. **B. Edge bias;** The object is counted in biased frames 5 and 8. The object, however, is counted in unbiased frame 8 only. **C. 3-D unbiased probe;** Unbiased dissector counting frames showing inclusion (green) and exclusion (red) line (2-D) and inclusion and exclusion (red) planes (3-D).

The dissector principle avoids the edge effect by using unbiased frame and counting rules. (H. J. G. Gundersen, 1977; West, 1993a) The edge problem is that the object in the edge of the probe can be counted more than one time using a biased frame (Figure 6,

B).(Mandarim-de-Lacerda, 2003; West, 1993a) The 3-D disector probe consists of inclusion plane and exclusion plane. On the disector plane, the upper and right plane is inclusion plane while the lower and left plane is exclusion plane. The exclusion plane extends to infinity above the left plane and below of the right plane. In addition to these planes, the top and bottom of the 3-D probe are covered with a guard volume, which avoids an artificial effect from cutting the section with blade. This is called “capping” or cutting the neurons on half. In unbiased counting rules, the objects within the 3-D probe or touching including the inclusion bars must be counted, while the object out of the 3-D probe or touching excluding bars must not be counted. For instance, the object in B of Figure 5 can be counted 2 times within biased probe but only one time within unbiased probe (Figure 6, B). All of the object counted should be not within the guard volume and the top of the object should be focused within 3-D probe through the microscope.

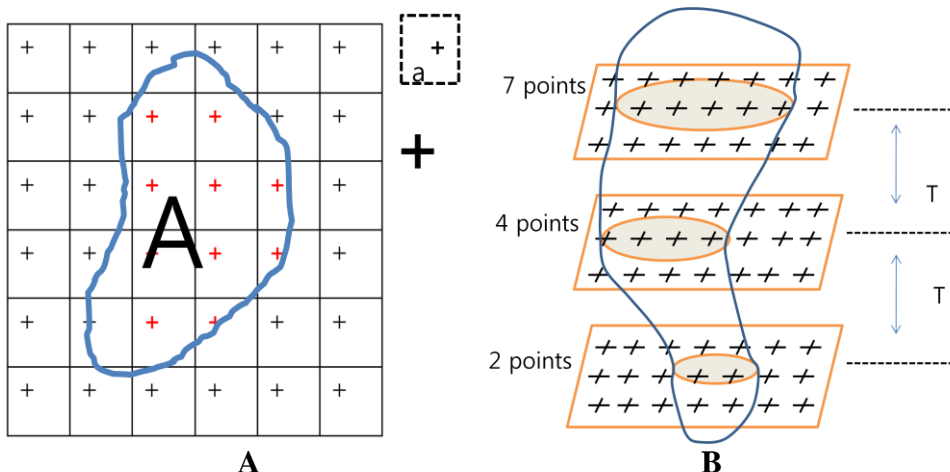


Figure 7. Cavalieri-point counting method to estimate volume; A. Counting the sum of dimensionless points (arrow) spaced apart by area, a , and placed at random over profiles leads to unbiased estimates of the expected profile area. **B.** Combination of Cavalieri volume and point counting to estimate total volume of an arbitrarily shaped object.

Third, the Cavalieri-point counting method can estimate the volume of arbitrary shaped biological objects. (Kubinova & Janacek, 2001) Bonaventura Cavalieri demonstrated that the volume of arbitrary shaped objects can be estimated with the sum of the area selected randomly throughout the whole object and the distance between selected planes (Figure 7). (Mandarim-de-Lacerda, 2003) Based on the Cavalieri method, Thomson developed cavalieri-point counting in 1930. (Thomson, 1930) Instead of the profile area, this new technique uses the number of selected point within the profile area. Points are placed in a systematic-random manner throughout the reference area as well as sections. The formula to estimate volume of object K is

$$\text{Volume } K = \sum P \times a(p)$$

In this formula, P is the number of point hitting the reference area in each section and $a(p)$ is the area per point. This method is theoretically unbiased and need no mathematical

assumptions to estimate volume in biological object. Compare to conventional pixel counting method, the Cavalieri-point counting method is more efficient and precise in estimating the volume of biological objects such as cells, nuclei, and nucleoli. Also, this method has been seemed to have high Inter- and intra-rater reliability.(Kubinova & Janacek, 2001; Mandarim-de-Lacerda, 2003; Peter R. Mouton, 2002) Therefore, the Cavalieri-point counting method is unbiased and on accurate volume estimator in biological objects.

In summary, with the unbiased stereological method based on probability theory, researchers can accurately and efficiently estimate morphological and anatomical changes in biological samples. There several functions of unbiased stereology that reduce the variation, effort, and time. In analysis, first, systematic-random sampling is theoretically unbiased and increases the efficiency of stereological estimates. Second, the dissector principle based on probability theory overcomes bias in estimating accurate object numbers in defined biological reference areas. Third, the Cavalieri-point counting method can estimate the volume of arbitrary shaped biological object accurately and efficiently. Thus, neuroscientists use unbiased stereology to investigate the neurobiological changes of in brain.

Anatomical and cytoarchitectural changes are related to brain function.

Anatomical features are very important in the brain because specific brain regions are specialized for different functions.(Fabricius, Wortwein, & Pakkenberg, 2008) Cerebral

cortex is the outmost layer of the cerebrum (often referred to as grey matter) and is formed by neurons and unmyelinated fibers (and glia, etc.). The cerebral cortex is involved in higher brain functions including the generation of motor commands, sensory perception, conscious thought, spatial reasoning, and language in humans.(Haines & Ard, 2002; Kandel, Schwartz, & Jessell, 2000) Gross anatomical structures, such as gyri and sulci, are landmarks in the definition of functional areas. For example, the motor cortex is located in the precentral gyrus and the sensory cortex is located in postcentral gyrus. In addition to gross anatomy, the cytoarchitecture of the brain also differs according to function. Generally the laminar pattern of the cerebral cortex consists of six layers which are the molecular layer I, the external granular layer II, the external pyramidal layer III, the internal granular layer IV, the internal pyramidal layer V, and the multiform layer VI. Some of functional cortices have their own characteristics in the laminar pattern that are exceptions to this uniformity. Sensory cortices, including the primary visual cortex, are likely to contain very prominent layer IV while motor cortices, such as the primary motor cortex, lacks a layer IV.(Kandel, et al., 2000) Also, the specific type of neurons take a critical role in particular brain areas. Dopaminergic(DA) neurons in the substantia nigra(SN) control the balance between the direct and the indirect pathways in the basal ganglia. Pyramidal neurons are the principal neural cell for vision guided motor function in the corticospinal tract.(Salimi, Friel, & Martin, 2008) Thus, anatomical and cytoarchitectural features are related to the function of specific brain areas.

Anatomical changes of a specific brain area reflect the associated brain function. Volume of nuclei or brain area is major anatomical parameter. Change in the volume of a specific area affects the function related to the brain region. Many neurological diseases cause cerebral atrophy, a reduction in the cerebrum decreases significantly. Volume reduction is associated with cognitive and motor dysfunction. For example, stroke survivors have about 10 times the risk for dementia compared to age-matched non-stroke controls.(Kokmen, Whisnant, O'Fallon, Chu, & Beard, 1996) Cognitive dysfunction after stroke is associated with both medial temporal atrophy (MTA)(Fein, et al., 2000; Gainotti, et al., 2004; Jokinen, et al., 2004) and global atrophy.(Jokinen, et al., 2005; Mok, et al., 2005) MTA is a powerful predictor of memory function when memory is assessed 2 years subsequent to stroke.(Firbank, et al., 2007) In stroke patients with cognitive impairment, gray matter volume in thalamus has been shown to decrease significantly while only small volume reduction of gray matter volume has been demonstrated in the cingulated gyrus, and frontal, temporal, parietal, and occipital lobes.(Stebbins, et al., 2008) Altered motor function is also associated with brain volume changes. In magnetic resonance imaging (MRI) study with older adults, difficulties in gait (such as shorter steps and longer support time) are related to reduced volume in sensorimotor regions and frontoparietal regions within the motor speed domain.(Rosano, et al., 2008) In addition to anatomical changes, cytoarchitectural variation (such as neuronal number and complexity of dendritic formations) may also influence brain

function.

Alteration of number or density may influence brain function. The decrease of neuronal number in specific brain area may cause brain deficits and has significant functional implications.(Chen, King, Lee, Sedtal, & Smith, 2006) Reduction of the neurons in the primary visual cortex are associated with visual perception deficits (e.g., 25% reduction in Brodmann's area 17).(Dorph-Petersen, et al., 2008) Fewer DA neurons in the SN causes both motor and cognitive deficits because the SN regulates the flow of information to cerebral cortex in cortico-basal ganglia circuits and the basal ganglia-brainstem (BG-BS) system.(Ahmad, Park, Radel, & Levant, 2008; Ahmad, Park, Stenho-Bittel, & Lau, 2009; Haines & Ard, 2002; Kalbe, et al., 2008; Kandel, et al., 2000; Klepac, Trkulja, & Relja, 2008; Lin, Sullivan, Wu, Katak, & Winstein, 2007) The decrease of neurons in amygdala may causes difficulties in emotion recognition and extraction in people with autism.(Clark, Winkielman, & McIntosh, 2008; Gross, 2005; Hobson, Ouston, & Lee, 1988; Kuusikko, et al., 2009; Loveland, et al., 1997) The amygdala plays important role in generation and recognition of emotions such as fear and has been consistently associated with the pathology of autism.(Baron-Cohen, et al., 2000) In autism, the total number of neurons in the amygdala is significantly fewer than in to control group.(Schumann & Amaral, 2006)

The change of dendritic complexity is also an indicator of brain function. Dendrites are important structures of neurons used to receive communication from other

neurons.(Kandel, et al., 2000) Increase of dendritic length and branching can be interpreted as strengthening the neural network related to a specific activity.(Bulinski, et al., 1998; Korkotian & Segal, 2000) Training rats using a Morris water maze (MWM) for spatial learning generated increased dendritic length and branching of layer III pyramidal cells in the occipital cortex, which play an important role in visual-spatial function. It also increased the number of astrocytes, playing important role in learning and memory, in the CA3 area of the hippocampus.(Jahanshahi, Sadeghi, Hosseini, Naghdi, & Marjani, 2008; Kolb, Cioe, & Comeau, 2008) Visual activity alters the visual cortex,(Greenough, Juraska, & Volkmar, 1979) whereas motor learning activities contribute to increased dendritic length and branching in layer V pyramidal cells of the motor cortex (specifically in the forelimb area with tray-reaching, single-pallet retrieval, and string-pulling tasks).(Greenough, Larson, & Withers, 1985; Kolb, et al., 2008; Withers & Greenough, 1989) Functional improvement due to learning is associated with area-specific dendritic growth related to the functional activity.(Kolb, et al., 2008; Kolb, Gibb, & Gorny, 2003; Whishaw, Alaverdashvili, & Kolb, 2008)

In conclusion, neuroscience research support that anatomical and cytoarchitectural features are associated with brain functions. This structure-function relationship suggests a specific brain structure is related to a particular brain function. Changes in anatomical and cytoarchitectural features in a specific area affect specific brain functions. Smaller

sensorimotor regions and frontoparietal regions are related to difficulties in gait. Motor function improvements due to functional activity are associated with dendritic length and branching in layer V pyramidal cells in the motor cortex for forelimb. Because of the structure-function relationship, anatomical and cytoarchitectural changes can be neurobiological effect related to functional improvement in rehabilitation. In neuroscience, stereology is effective and accurate method to quantify neurobiological effect in anatomical or cytoarchitectural feature. In rehabilitation, OT has an important role to improve the client's function in occupational performance by inducing neurobiological changes as a consequence of the intervention employed. Anatomical and cytoarchitectural effects of intervention support the functional effect or provide possibility of functional improvement. Thus, stereology may be a bridge the study of neuroscience and OT practice.

Stereology informs translation from neuroscience to OT.

<i>AREAS OF OCCUPATION</i>	<i>CLIENT FACTORS</i>	<i>PERFORMAN CE SKILLS</i>	<i>PERFORMAN CE PATTERNS</i>	<i>CONTEXT AND ENVIRONMENT</i>	<i>ACTIVITY DEMANS</i>
Activities of Daily Living(ADL)	Values, Beliefs, and Spirituality	Sensory Perceptual Skills	Habits Routines Roles Rituals	Cultural Personal Physical Social Temporal Virtual	Objects Used and Their Properties Space Demands Social Demands Sequencing and Timing Required Actions Required Body Function Required Body structures
Instrumental Activities of Daily Living(IADL)	Body Functions	Motor and Praxis Skills			
Rest and Sleep	Body Structures	Emotional Regulation Skills			
Education		Cognitive Skills			
Work		Communication and Social Skills			
Play					
Leisure					
Social Participation					

Figure 8. Aspect of Occupational Therapy's Domain. This figure represents the domain of occupational therapy and is included to allow readers to visualize the entire domain with all of its various aspects. No aspect is intended to be perceived as more important than another. (modified AOTA(Roley, et al., 2008))

OT has vital connections and a long history of informing practice with neuroscience.(Farber, 1989) Neuroscience provides important related knowledge regarding human occupation by helping the therapist to understand these performance skills from the perspective of brain function. In the OT paradigm, humans have needs and motivation to participate in meaningful tasks and activities. This is referred to as occupational nature or needs.(Kielhofner, 1997) When an individual participates in occupation, six major domains of OT must be considered.(Roley, et al., 2008) These domains include area of occupation, client factors, performance skills, performance patterns, context and environment, and activity demands (Figure 8). Among the biopsychosocial domains, performance skills are the brain. According to the OT framework, performance skills are observable, concrete, goal- functions

most related to neuroscience because they are represented by neural processes in directed actions clients use to engage in daily life occupations.(Roley, et al., 2008) The categories of these skills include motor and praxis skills, sensory-perceptual skills, emotional regulation skills, cognitive skills, and communication and social skills (Figure 8).(Roley, et al., 2008) Specific brain structures and neural circuits are associated to these performance skills. Performance skills are the individual's demonstrated abilities to participation in their occupation. Without appropriate performance skills, an individual has difficulties to participate in occupation. The alteration of neurological status may affect performance skills critical to participate in occupation because neural process and structures are associated to performance skills. Therefore, knowledge from neuroscience is primary for OT practitioners to understand how neurological status affects performance skills in occupational participation.

Neuroscience provides fundamental knowledge how neurological changes influence performance skills. Negative changes of neurological status are neurological disorders, such as stroke, traumatic brain injury, PD, and Alzheimer disease (AD). These disorders can cause difficulties participating in occupation due to disruption of neural processes related to performance skills. Through neuroscience knowledge, occupational therapists can understand which performance skills may be influenced by a specific neurological disorder. For example, people with left middle cerebral artery (MCA) infarct may exhibit aphasia and motor deficits in their right extremities because left MCA has trouble with supplying blood to left brain

areas playing roles in verbal expression (broca's area: brodmann 44 and 45) and in motor control of right extremities (primary motor area: brodmann 4). Lack of blood causes disorder in these brain areas. Aphasia limits communication and social skills and right side motor deficits impair motor performance and/or praxis skills. The memory deficits associated with AD can be explained by the atrophy of the hippocampus (a negative change of neurological status) and reflect diminished cognitive skills (a performance skill). Because of these deficits in performance skills, an individual with neurological disorder may have difficulties with participation in areas of occupation such as activities of daily living, education, rest and sleep, work, play, leisure and, social participation. Neuroscience provides knowledge to explain why people with left MCA stroke have difficulties in motor and communication skills and why people with AD have poor memory.

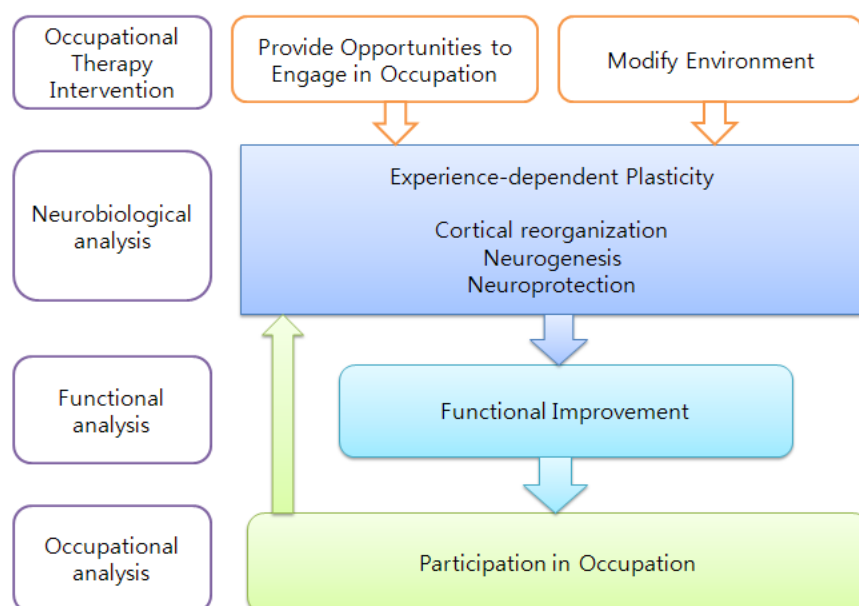


Figure 9. Dynamics of employing occupation as therapy in neurological system.

In addition, neuroscience researches demonstrated non-pharmacological therapeutic interventions induce positive changes in neurological status. The adaptive ability of central nervous system is plasticity. The existing studies strongly support the neurological system possesses the remarkable capacity to change their function and structure in response to a variety of experiences including behavioral training and enriched environment. Occupational therapists improve performance skills of people with neurological disease to help them to participate in occupation. The OT does so by providing opportunities to engage in the occupation and by modifying environments to support that engagement. Occupational therapists believe that these two therapeutic approaches induce positive neurobiological changes in specific brain areas related to the affected performance skill (figure 9). (Lohman & Royeen, 2002) Many neuroscience studies reveal that these therapeutic approaches induce positive changes in the nervous system in animal models. For example, enriched environments (EE) alter the neurological status. In animal studies, generally EE are comprised of bigger housing cages, with objects to interactivity, a running wheel, and tunnels that are changed occasionally to motivate animal exploration. (Bezard, et al., 2003; Laviola, Hannan, Macri, Solinas, & Jaber, 2008; Rosenzweig & Bennett, 1996) Several studies on effects of EE on neurological disorders, such as PD, (Bezard, et al., 2003) Huntington's disease, (Nithianantharajah, Barkus, Murphy, & Hannan, 2008; Spires, et al., 2004; van Dellen, Blakemore, Deacon, York, & Hannan, 2000) and Alzheimer's disease, (Berardi,

Braschi, Capsoni, Cattaneo, & Maffei, 2007; Gortz, et al., 2008; Jankowsky, Xu, Fromholt, Gonzales, & Borchelt, 2003) revealed that EE enhanced experience-dependent plasticity in transcription of specific genes, synaptogenesis, and adult neurogenesis in the rat model.(Laviola, et al., 2008) The knowledge from animal studies does not directly translate to particular recommendations for OT practice. However, knowledge from neuroscience studies in animal models provides neurobiological phenomenon related to functional recovery and to identify fundamental principles helps to guide the optimization of therapeutic approaches.(Kleim & Jones, 2008)

Stereology is one of neurobiological analysis to inform translation from neuroscience to OT. Neuroscience provides various analysis techniques to investigate neurobiological changes to study neurobiological recovery and fundamental principles of neural plasticity. Various neurobiological techniques, including neuroimaging, electroneurophysiology, neurochemistry, stereological, and genetics, demonstrate neurobiological changes due to therapeutic intervention. For example, electroneurophysiological techniques can detect change in functional areas of the cerebral cortex and neurochemical techniques can demonstrate a change in the amount of neurotransmitters in the brain. Stereology can investigate the change of anatomical and cytoarchitecture in volume, number, length, and surface area. Based on structure-function relationship, stereology informs translation from neuroscience to OT in three ways. First, stereology can be used to demonstrate anatomical

and cytoarchitectural features related to brain functions associated with changes in performance skills.(Botteron, Raichle, Drevets, Heath, & Todd, 2002; Sajdel-Sulkowska, et al., 2005) Second, stereology can document anatomical and cytoarchitectural characteristics of neurological disorders.(Du, et al., 2004; Grady, et al., 2003; Heckers, Heinsen, Geiger, & Beckmann, 1991) These characteristics may explain difficulties in which performance skills decreases from neurological disease or disorder. Third, stereological research demonstrates experience-dependent structural plasticity related to functional improvement supporting OT practice. Coupling stereological and functional outcome reveals that neurological plasticity which may indicate functional improvement. Therefore, stereology informs translation from neuroscience to OT by analyzing anatomical or cytoarchitectural features related to performance skills in neurological disease or disorder.

Stereological method can be used to demonstrate anatomical and cytoarchitectural features related to performance skills. Number and volume are most frequently used in stereological studies as a key parameter. These parameters in anatomical or cytoarchitectural features are associated with performance skills including motor, cognition, social, and communication skills. For example, the correlation is significantly positive in a rat model between the number of purkenje cells in the cerebellum and motor behavior, such as rotorod test and parallel bartest.(Goodlett & Lundahl, 1996; Sajdel-Sulkowska, et al., 2005) Purkenje cells in the cerebellum play crucial roles in motor skills including coordination. In cognitive

skill, quantitative MRI study using stereological method demonstrated that the volume of white matter has significantly positive correlation with Cognitive Index Score(CIS) and Figural Memory Test (FMT) in multiple sclerosis patients.(Edwards, et al., 2001) Also, emotion and cognitive skills are related to these stereological parameters. In quantitative MRI studies with human subjects, hippocampus(Mervaala, et al., 2000) and subgenual prefrontal cortex (SGPFC)(Botteron, et al., 2002) volume were smaller in people with depression than in control. In stereological study with postmortem brain in bipolar disorder, reduced neural density (16%~22%) in layer III and reduced pyramidal cell density (17%~30%) in layer III and V were demonstrated in dorsolateral prefrontal area 9.(Rajkowska, Halaris, & Selemon, 2001) Anatomical changes of the limbic system, including the medial orbitofrontal cortex, hippocampus, amygdale, and prefrontal cortex, are associated with mood disorder.(Botteron, et al., 2002; Bremner, et al., 2000; Mervaala, et al., 2000; Rajkowska, et al., 2001) Nuclei of the thalamus connected to the limbic system, such as the mediodorsal and anterorventral/anteromedial nuclei, are associated with emotional function.(Young, Holcomb, Yazdani, Hicks, & German, 2004) The number of neurons in these nuclei of the thalamus demonstrates influences on emotion disorders such as depression. People with major depression have 37% more neurons in the mediodorsal and 26% more neurons in anterorventral/anteromedial nuclei.(Young, et al., 2004) Language is an important component in communication skills. Wernicke's and Broca's areas, two major brain regions involved in

language, are proportionally larger in females than in males. In stereological research with postmortem human brain, females have 29.8% larger superior temporal cortex and 20.4% larger Broca's area, which may explain superior language skills in females.(Harasty, Double, Halliday, Kril, & McRitchie, 1997)

Stereology may be used to investigate anatomical or cytoarchitectural characteristics of neurological disease and disorder, which can help occupational therapists to understand why patients have difficulties in performance skills due to neurological disorders such as stroke, TBI, PD, Huntington disease, ALS, Alzheimer's disease, autism, Down's syndrome, and schizophrenia. For instance, design-based cell counting reveals neuronal number of CA2/3 in hippocampus significantly decreases after TBI in rat with fluid percussion brain injury, providing an explanation for why this form of traumatic brain injury induces spatial memory dysfunction.(Grady, et al., 2003) After TBI, both humans(Levine, et al., 2002) and experimental animals(Pierce, Smith, Trojanowski, & McIntosh, 1998; D. H. Smith, et al., 1997) typically have impairments of memory and cognitive function. In human postmortem study with stereological method, the characteristics of neuronal loss in the thalamus are associated with severity of injury assessed by Glasgow Outcome Scale (GOS).(Maxwell, MacKinnon, Smith, McIntosh, & Graham, 2006) The number of thalamic neurons after TBI is typically reduced significantly in executive and cognitive nuclei, reduced to a lesser degree in somatosensory nuclei, and reduced the least in limbic motor nuclei. Mediodorsal thalamus

is considered as modulator of cognitive functions in the prefrontal cortex.(Mitchell, Browning, & Baxter, 2007) In the mediodorsal parvocellular nuclei (MDpc), 14.6% neuronal loss of occur in moderately injured, 19.6% in severely injured, and 36.6% in a vegetative condition.(Maxwell, et al., 2006) Not only TBI but also other neurological disorders, including AD and schizophrenia, have stereological characteristics related to performance skills.

AD is confirmed by presence high densities of amyloid plaques,(Mirra, Hart, & Terry, 1993) neurofibrillary tangles,(Braak & Braak, 1997) severe cortical atrophy,(P. R. Mouton, Martin, Calhoun, Dal Forno, & Price, 1998) and neuronal loss especially in the hippocampus.(Du, et al., 2004; Price, et al., 2001) Unbiased stereology quantifies the volume of cortical area by using point counting and the Cavalieri principle(H. J. Gundersen & Jensen, 1987; H. J. Gundersen, et al., 1999) and reveals cortical atrophy in AD is 20 to 25% greater than in a control group.(P. R. Mouton, et al., 1998) Severe cortical atrophy, an anatomical feature, is highly correlated to cognitive performance on Mini Mental State Examination(MMSE).(P. R. Mouton, et al., 1998) AD is among the most devastating neurological disorders that affect the hippocampus.(Lange, et al., 2004) Neuronal loss in the hippocampus and the entorhinal cortex is not a feature of normal aging but substantial in people with AD.(Gomez-Isla, et al., 1996; Juottonen, Lehtovirta, Helisalmi, Riekkinen, & Soininen, 1998; Kordower, et al., 2001; West, 1993b; West, Coleman, Flood, & Troncoso,

1994) Stereological investigation demonstrates very mild AD group has 36% fewer neurons in the entorhinal cortex and 45% less in CA1 than control.(Pierce, et al., 1998) In addition to AD, schizophrenia also has devastating effects on the hippocampus.(Lange, et al., 2004)

In schizophrenia, the total volume of the hippocampus is about 5% smaller than controls.(Dwork, 1997; Heckers, et al., 1998; McCarley, et al., 1999) Several studies reported abnormality of the hippocampus in schizophrenic brain,(Bogerts, 1997; Dwork, 1997) but neuronal loss of the hippocampus is controversial in postmodern stereological studies.(Falkai & Bogerts, 1986; Heckers, et al., 1991) However, it seems questionable that any one part of brain causes the wide range of symptoms in schizophrenia.(Thune & Pakkenberg, 2000) Thus, investigators have been focusing on the thalamus, which is the main relay of cortical sensory projections and has an important role in the basal ganglia and the limbic system.(Groenewegen, Wright, & Uylings, 1997; Thune & Pakkenberg, 2000) Another interested nucleus of the thalamus involved in schizophrenia is the mediodorsal thalamic nucleus (MD). The MD has reciprocal projections with the prefrontal cortex (PFC).(Groenewegen, et al., 1997; Thune & Pakkenberg, 2000) A core deficit of schizophrenia is deficit in working memory which is one of the function of the PFC.(Barch, Sheline, Csernansky, & Snyder, 2003; Manoach, 2003; Pae, et al., 2008; Silver, Feldman, Bilker, & Gur, 2003) Stereological studies demonstrate the neuronal number of the MD in schizophrenia decreases by 30%(Thune & Pakkenberg, 2000) and the somal volume of

pyramidal neurons in schizophrenia decreases by 9.2%.(Pierri, Volk, Auh, Sampson, & Lewis, 2001) In addition, other brain areas, such as the limbic system,(Pakkenberg, 1990) the lateral geniculate nucleus,(Dorph-Petersen, et al., 2009) and the basal ganglia,(Roberts, Roche, & Conley, 2005) showed abnormality of stereological features. Therefore, stereology connects the anatomical and cytoarchitectural characteristics of neurological disorders with impaired performance skills, which helps translate neurobiological features to occupational performance. These characteristics provide fundamental reasons for why stereological analysis could be an outcome evaluation of therapeutic intervention in neurological disorders.

Stereological research demonstrates experience-dependent structural plasticity related to functional improvement in OT practice. In a rat with focal sensorimotor cortical damage, the effect of acrobatic training can be evaluated by stereological method. Stereological analysis demonstrated that the acrobatic exercise increases cortical and dendritic volume in the hindlimb representative sensorimotor cortex area (HLsmc).(Chu & Jones, 2000) The training group also showed better performance in the footfault performance test. In this study, stereological analysis reveals behavioral training after cortical injury enhances structural plasticity in relevant brain areas; experience-dependent structural plasticity.(Chu & Jones, 2000) In other stereological studies in rat model, behavioral training induces changes of dendritic complex. Functional activities, such as tray-reaching, single-pellet retrieval, string-pulling, and T-maze task, induce experience-dependent changes in

dendritic length and branching related to functional activities.(Kolb, et al., 2008; Kolb, et al., 2003; Whishaw, et al., 2008) Stereological analysis is critical for demonstrating therapeutic effects, especially in neurodegenerative diseases, because neuronal loss or atrophy is main feature of these types of diseases. Stereological analysis shows that long-term physical exercise preserves the neuronal loss in Purkinje cell of the cerebellum in rats. Aged-match controls have 11% fewer Purkinje cells and 9% smaller Purkinje cell soma volume than the exercise group.(Larsen, Skalicky, & Viidik, 2000) In a previous study on PD, unbiased stereological cell counting evaluated the number of DA neurons in the SN after endurance exercise training.(Ahmad, et al., 2009) Stereological research in neuroscience supports OT practice in neurological disorder demonstrating experience-dependent plasticity.

However, stereology has limitation to translate from neuroscience direct to OT practice. Investigators cannot use direct stereological method to demonstrate neurobiological feature in human except in postmortem study. Some of MRI studies in human used stereological measurement (cavalieri-point counting) to detect the change of volume(Bas, et al., 2009; Keller, Baker, Downes, & Roberts, 2009; Kempton, et al., 2009; Mazonakis, et al., 2008) but it is impossible to count neurons or to detect the change of dendritic complex in live human subjects because of practical and ethical issues.(Cramer & Riley, 2008) Therefore, most of stereological studies are in animal model(Ahmad, et al., 2008; Sanchez, et al., 2008) or in postmortem human.(Byne, Hazlett, Buchsbaum, & Kemether, 2009; Dorph-Petersen, et

al., 2009; Hercher, et al., 2009) Even though occupational therapists cannot literally apply knowledge from these stereological researches in animal model or postmortem human to OT practice, these fundamental studies provide basic knowledge of neurological disease, contribute to modify the interventions to more effective in humans, and support evidence to design well built human studies. Fundamental principles of neurological recovery and neural plasticity from these studies help therapists to optimize OT practice.(Kleim & Jones, 2008)

In conclusion, stereology informs translation from neuroscience to OT practice. OT has a crucial relationship with neuroscience. Performance skills are the essential domain of OT practice and performance skills are governed by neurological processes. The cellular elements supporting these neurological processes may be altered through experience and training. Performance skills are essential to participate in occupation. With insufficient performance skills due to neurological disorders, an individual may have trouble to participate in occupation. Therefore, occupational therapists must consider implications of neuroscience knowledge in order to understand human occupation, especially when neurological disorders are a factor. Stereology is one means to establish a bridge between OT practice and neuroscience principles, because stereology can quantify anatomical and cytoarchitectural features related to performance skills. There are three ways stereology translates from neuroscience to OT. First, stereology demonstrates the relationship between anatomical and cytoarchitectural features and performance skills. Second, stereology reveals

anatomical and cytoarchitectural characteristics of neurological disorder. Third, stereological research demonstrates experience-dependent structural plasticity related to functional improvement in OT practice. Stereology has limitation to translate from neuroscience direct to OT practice because investigators cannot use direct stereological method in human studies. However, stereological researches in neuroscience provide basic knowledge of neurological disease, contribute to modify the interventions to more effective in humans, and support evidence to design well built human studies. Therefore, stereology can make the connection between neuroscience and OT.

Conclusion

Stereology is an interdisciplinary field focused on analyzing biological tissue with the three-dimensional interpretation of planer sections by using estimating method and mathematically unbiased sampling. The primary parameters in stereology are cell volume, cell number, cell surface area, and process length. However, classical assumption-based geometry cannot be applied directly to biological investigation because biological objects have random variation. Modern stereology overcomes the limitation of assumption-based geometry with probability theory. There are two main sources of error; systemic and non-systemic error. For an accurate estimation of parameters in a biological object of interest, researchers need to minimize both systematic and nonsystematic variation through both

unbiased methodology and precise sampling. Therefore, researchers can use unbiased stereology to estimate changes in biological objects precisely, including changes in the cellular composition and complexity of the brain. Current methods of modern stereology overcome prior limitations of assumption- and model-based classical stereology.

With the unbiased stereological method based on probability theory, researchers can estimate morphological and anatomical changes in biological reference areas accurately and efficiently. There several beneficial methods of unbiased stereology that reduce variation, effort, and time. First, systematic-random sampling is theoretically unbiased and increases the efficiency of stereological estimate. Second, the disector principle based on probability theory overcomes bias in estimating accurate object number in defined biological reference area. Third, the Cavalieri-point counting method can estimate the volume of arbitrary shaped biological object accurately and efficiently. Thus, neuroscientists use unbiased stereology to investigate the neurobiological changes, such as anatomical and cytoarchitectural features, in brain.

Anatomical and cytoarchitectural features are related to function in the brain. Changes in anatomical and cytoarchitectural parameters, such as volume, number, and length, affect specific brain function related to the brain area. Neuronal and volume loss of specific brain area causes decline of the brain functions such as motor and cognitive and also functional improvement is associated to anatomical and cytoarchitectural features.

Occupational therapists provide treatment to improve functions for participation of occupation in neurological disorder. The functional improvements in neurological disorder reflect neurobiological changes because functional difficulties, such as motor cognitive disorder, are due to neurological disturbances. Thus, combination of two kinds of evidence, neurological changes and functional improvement, provide fundamental evidence for OT intervention in neurological disorder.

In conclusion, stereology is one scientific method available to aid in translating neuroscience principles to OT practice. The unbiased stereological method improves accuracy and efficiency in data collection of anatomical and cytoarchitectural changes of brain with neurological disorder in neuroscience. OT has vital relationship with neuroscience because performance skills are critical for participating in occupation and performance skills are based upon neural processes that can be modified through experience or training. OT intervention improves functions for participation of occupation through neurological improvement in neurological disorder. Stereological analysis shows neurological changes related to function. It demonstrates the relationship between performance skills and neurological features, neurological characteristics of neurological disorders, and neurological effect of therapeutic interventions demonstrating experience-dependent structural plasticity. Even though most of stereological studies are in animal model and in postmortem human because of practical and ethical issues, stereology provides fundamental knowledge to

support OT theory and practice. Therefore, stereology informs translation from neuroscience to OT based on structure-function relationship in performance skills and experience-dependent neural plasticity.

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