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Doctoral Thesis

Regional and Inter-Laminar Differences of Postnatal Development of Pyramidal Cells in the Primary Visual, Visual Association, and Prefrontal Cortex of Macaque Monkeys

（マカカ属サル一次視覚野、視覚連合野、前頭前野における錐体細胞の領野および層特異的生後発達）

Tomofumi OGA
Laboratory for Cognitive Neuroscience,
Graduate School of Frontier Biosciences, Osaka University

Supervisor
Professor Ichiro FUJITA

Thesis Committee
Professor Nobuhiko YAMAMOTO
Professor Akihiko OGURA
Associate Professor Keiko TOMINAGA-YOSHINO

March 2017
Abstract

Pyramidal cells in the primate cerebral cortex, particularly those in layer III, exhibit regional variation in both the time course and magnitude of postnatal growth and pruning of dendrites and spines. Less is known about the development of pyramidal cell dendrites and spines in other cortical layers. Here I studied dendritic morphology of layer-V pyramidal cells in primary visual cortex (V1, sensory), cytoarchitectonic area TE in the inferotemporal cortex (sensory association), and granular prefrontal cortex (Walker’s area 12, executive) of macaque monkeys at the ages of 2 days, 3 weeks, 3.5 months, and 4.5 years. I found that changes in the basal dendritic field area of pyramidal cells were different across the three areas. In V1, field size became smaller over time (largest at 2 days, half that size at 4.5 years), in TE it did not change, and in area 12 it became larger over time (smallest at 2 days, 1.5 times greater at 4.5 years). In V1 and TE, the total number of branch points in the basal dendritic trees was similar between 2 days and 4.5 years, while in area 12 the number was greater in the adult monkeys than in the younger ones. Spine density peaked at 3 weeks and declined in all areas by adulthood, with V1 exhibiting a faster decline than area TE or area 12. Estimates of the total number of spines in the dendritic trees revealed that following the onset of visual experience, pyramidal cells in V1 lose more spines than they grow, whereas those in TE and area 12 grow more spines than they lose during the same period. These data provide further evidence that the process of synaptic refinement in cortical pyramidal cells differs not only according to time, but also location within the cortex. Furthermore, given the previous finding that layer-III pyramidal cells in all these areas exhibit the highest density and total number of spines at 3.5 months, the current results indicate that pyramidal cells in layers III and V develop spines at different rates.
Acknowledgement

First of all, I would like to show my sincere gratefulness to my supervisor, Prof. Ichiro Fujita, for his continuous support throughout my Bachelor, Master, and Ph.D. course. With his enthusiasm and patience, he has helped me to make this thesis possible. I would like to thank Dr. Guy N. Elston for his continuous encouragement. He helped me learn expertise in anatomical sciences. I am very grateful to Tsuguhisa Okamoto for his assistance throughout the project. I thank Dr. Tomoyoshi Urakubo. He trained me to electron microscopic observation. I thank Dr. Noritaka Ichinohe, Dr. Tetsuya Sasaki, and lab members for their support at National Center of Neurology and Psychiatry (NCNP). Dr. Ichinohe provided me an opportunity of applying the experimental technique to marmosets. Dr. Sasaki took over my work at NCNP. I thank Ryosuke Takeuchi, Dr. Shuntaro Aoki, Dr. Mikio Inagaki, and Dr. Hiroshi Tamura for the scientific discussions and criticism. I would like to thank Ms. Kyoko Yoshimura and Ms. Takako Tanaka, who helped me by their continuous official support. My gratitude also goes to Dr. Nobuhiko Yamamoto, Dr. Akihiko Ogura, and Dr. Keiko Tominaga-Yoshino, for serving the dissertation committee.

Part of this dissertation has been published in the following articles.


## Abstract

Abstract

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Curriculum vitae
Chapter 1. General Introduction

This dissertation reports on regional and inter-laminar differences in the developmental profile of cortical pyramidal cell morphology in macaque monkeys (Macaca fascicularis). The purpose of the study was to understand how the developmental profile of pyramidal cells varies among cortical areas and between cortical layers and how different the dendritic structure is within a cortical area. This first chapter of the dissertation presents the background, problem statement, purpose, significance and outline of the study.

1.1. Background of the problem

Dendrite development is a crucial process in the maturation of neural circuit. Pyramidal cells, which is ‘output’ cells in the cortical areas, have 80% of excitatory synapses on dendritic spines (Figure 1.1). Spines are small protrusions sprouting from dendrites (DeFelipe, 2010 for review). Petanjek and colleagues (2008) reported that layer III pyramidal cells in human Brodmann’s area (BA; Brodmann, 1909) undergo development in two stages. The dendrites are less developed in length at birth in layer III than in layer V. Within one month, dendrites in layer III rapidly grow and catch up to the size of dendrites in layer V. After this initial growth, layer III neurons maintain their size during the 3 to 16 months after birth (steady period). At this point (around age 2.5 years), the second growth stage begins. The first stage of the dendrite development corresponds an onset of the stimulus. Around the second stage, human grow cognitive functions (Petanjek et al., 2011).

Figure 1.1: Morphology of pyramidal cells. The pyramidal cell is characterized by its pyramid-like shape. The pyramidal cell has an apical dendrite which grows toward the cortical surface and basal dendrites which radially spread out from the cell body. The dendrites have small protrusions on its shaft—spine which receives synaptic input from an axon.

Dendritic growth and spinogenesis both contribute to net change of synaptic input within a single cell. Rakic and colleagues (1986) investigated synapse density in the broad region of monkey cerebral cortex by electron microscopy (primary visual: Bourgeois et al., 1989; Bourgeois & Rakic, 1993;
somatosensory: Zecevic & Rakic, 1991; Motor: Zecevic et al., 1989; prefrontal: Bourgeois et al., 1994). They argued that overproduction and pruning of synapses occur concurrently across cortical areas. Synapse density peaked 2-4 months after birth and then decreased. The series of the study is not enough to understand the development of the neural circuits, because neurons, the core component of the circuit, has area specific dendrite development strategy (Boothe et al., 1979; Anderson et al., 1995; Jacobs et al., 1997, 2001; Travis et al., 2005; Petanjek et al., 2008), and then the total number of synaptic input on a single cell changes in different manners across cortical areas (Elston et al., 2009; 2010). In the macaque primary visual cortex, pyramidal cells grow their dendrites prenatally, then they prune it after birth (Boothe et al., 1979; Elston et al., 2010), while in association cortices, they continue to grow dendrites after birth through infant to adolescence (Anderson et al., 1995; Elston et al., 2009; 2010). The growth of dendrites provides synaptic contact sites to the cell. Although the estimation of the total number of spines on a pyramidal cell has a peak at 3.5 months of ages as the synapse density peaks, dendritic growth in the association cortices supports elongated synaptogenesis.

Non-human primate studies, which support brain development study in human, is needed due to limitations of human brain study. Technical and ethical matters limit human brain study so that noninvasive techniques such as electroencephalography and magnetic resonance imaging are used in the most of the developmental study, resulting in less informative than that of invasive techniques. In out of the limitations, there has been in-vivo electrophysiology study associated with epilepsy or brain tumor surgery. Although these studies have developed neuroscience (e.g. Penfield and Jasper, 1954), it is hard to carry out the series of developmental research. In contrast to human, rodents are used as a model animal, while their brain development is less featured than that of primates. For example, in primate synapses are overproduced at early developmental stage and then they are pruned, exhibiting overshoot type profile (human: Huttenlocher; macaque monkey: Rakic et al., 1984; marmoset: Missler et al., 1993). The amount of the spine reduction in the primate prefrontal cortex reaches seven times as many adult cells have. In contrast, rodents do not exhibit such greater reduction (Micheva and Beaulieu, 1996). For that reason, non-human primate, such as macaque monkeys and marmoset monkeys, are used to accumulate evidences at synapse level (Rakic et al., 1986; Missler et al., 1993), at cellular morphology (Anderson et al., 1995; Elston et al., 2009; 2010a; 2010b; 2011; Oga et al., 2013; Sasaki et al., 2015), in permeability of membrane (Luebke et al., 2015), and at behavior (Harlow and Mears, 1979).

In human and non-human primate, pyramidal cells are specialized among areas. Pyramidal cells in layer III have longer dendrites and many numbers of spines in high integration areas than in lower integration areas (Jacobs et al., 1999, 2001). This tendency clearly appears when comparing within a cortical pathway. In the visual system of macaque and marmoset, the dendritic field is wider, and the number of spines is larger in the higher visual cortices than in lower visual cortices (Elston and Rosa, 1996, 1997). The extent of the horizontal axon is also longer in TE (visual association) than in V1 (Tanigawa et al., 2005). The input resistance of the cell membrane is higher in V1 than TE and Walker's area 12 which has a direct projection from TE (Ishikawa et al., 2007; Maruyama et al., 2007; Amatrudo et al., 2012). The time constant of the cell membrane is longer in TE than V1. By these specializations, pyramidal cells of the association cortices have characteristics suitable for spatiotemporal input integration, while the primary visual cortex is suitable for sequential processing of sensory input.

The inter-areal specialization of layer III pyramidal cells is formed through area-specific development processes (Elston et al., 2009). When compared between V1 (primary sensory), TE (sensory association), and area12 (multisensory association), dendrites in association cortices grow after birth, while the dendrite field area shrinks in V1. The differences between areas are present even at birth, and regional differentiation proceeds by postnatal developmental changes. The spines are sparse at birth in all three areas, and the density rises over 3.5 months after birth. From birth to 3.5 months there is no regional difference, but after 3.5 months the spine pruning exceeds spinogenesis, resulting in a decline of spine density. In the process, the degree of spine pruning is greater in lower areas than in higher areas, so that the adult spine density differs between areas. In this way, the pyramidal cells mature under developmental process unique to the area, and they differentiate regionally.
1.2. Problem Statement

Human pyramidal cells in prefrontal cortex exhibit layer specific developmental profile. Yet, to date, there has been a little inter-laminar study that compares the pyramidal cell development across cortical areas. It is unknown whether and how the developmental profiles differ across cortical areas in infragranular layers remains unclear. While it occupies the attention whether the pyramidal cell development of monkey imitates it of human or not, available data can not answer the question. Perhaps a study that investigates pyramidal cell development of macaque monkeys between cortical areas with intracellular dye injection technique could reveal the problem.

1.3. Purpose of the study

The purpose of this study is to reveal the developmental profile of layer V neurons in the primary visual cortex as the primary sensory cortex, cytoarchitectonic area TE as a visual association area, and area 12 as an executive cortex of the cynomolgus monkeys (Macaca fascicularis). The macaque monkeys aging 2 days after birth to adult was chosen in this study. The pyramidal cell structure was visualized by injecting fluorescent dye after fixation. The full basal dendritic arbors were reconstructed and the dendritic spines density along the full extent was quantified. The dendritic field area, branching complexity were analyzed from the reconstructed dendrites. By combining the dendritic branching and the spine density, a total number of spines in “average cell” for the ages/areas is calculated. I visualized the developmental profiles of these morphological indices. As the results of this analysis, pyramidal cells in layer III and layer V exhibit the area and layer specific developmental profile.
1.4. **Significance of the Study**

The findings of this study will bring knowledge of pyramidal cell development in the infragranular layer to the primate research community. The present results provide anatomical evidence of the regional and inter-laminar difference of the pyramidal cell development in macaque monkeys.

1.5. **Outline of this thesis**

In this general introductory chapter, I have reviewed the reports on the development of the pyramidal cells in human and non-human primate and described the problem of this study. In this thesis, I address three issues in separate chapters.

In Chapter 2, I report that the pyramidal cells in layer V exhibit area specific developmental profile, and these profile are also layer specific.

In Chapter 3, I report that the dendrites of pyramidal cells in V1 are homogeneous within wider region of the area. The evidence supports that morphological difference of the cells represents cortical hierarchy.

In Chapter 4, I report that morphology of the pyramidal is different not only in dendritic branches and the number of spines but also spine structure. Spine head of pyramidal cells in association cortices are variable in size. The evidence indicates that pyramidal cells conduct plasticity of the neural network by not only a greater number of spines but also plasticity of each spine.

In Chapter 5, I provide general discussion and summary of the results presented in this thesis.
Chapter 1. General Introduction

References


Chapter 2. Postnatal Dendritic Growth and Spinogenesis of Layer-V Pyramidal Cells Differ between Visual, Inferotemporal, and Prefrontal Cortex of the Macaque Monkey

In this chapter, I show that the pyramidal cells in layer-V grow their dendrites at a different rate between cortical areas. The result adds further evidence that the pyramidal cells in cortical areas are characterized by different growth profile.

Pyramidal cells in the primate cerebral cortex, particularly those in layer III, exhibit regional variation in both the time course and magnitude of postnatal growth and pruning of dendrites and spines. Less is known about the development of pyramidal cell dendrites and spines in other cortical layers. Here I studied dendritic morphology of layer-V pyramidal cells in primary visual cortex (V1, sensory), cytoarchitectonic area TE in the inferotemporal cortex (sensory association), and granular prefrontal cortex (Walker’s area 12, executive) of macaque monkeys at the ages of 2 days, 3 weeks, 3.5 months, and 4.5 years. I found that changes in the basal dendritic field area of pyramidal cells were different across the three areas. In V1, field size became smaller over time (largest at 2 days, half that size at 4.5 years), in TE it did not change, and in area 12 it became larger over time (smallest at 2 days, 1.5 times greater at 4.5 years). In V1 and TE, the total number of branch points in the basal dendritic trees was similar between 2 days and 4.5 years, while in area 12 the number was greater in the adult monkeys than in the younger ones. Spine density peaked at 3 weeks and declined in all areas by adulthood, with V1 exhibiting a faster decline than area TE or area 12. Estimates of the total number of spines in the dendritic trees revealed that following the onset of visual experience, pyramidal cells in V1 lose more spines than they grow, whereas those in TE and area 12 grow more spines than they lose during the same period. These data provide further evidence that the process of synaptic refinement in cortical pyramidal cells differs not only according to time but also a location within the cortex. Furthermore, given the previous finding that layer-III pyramidal cells in all these areas exhibit the highest density and a total number of spines at 3.5 months, the current results indicate that pyramidal cells in layers III and V develop spines at different rates.

Part of this chapter has been published in the following article.

2.1. Introduction

Pyramidal cells in the primate cerebral cortex are characterized by different rates of growth and atrophy of both their dendrites and spines during development, resulting in marked phenotypic variation in their cellular structure among different areas in the mature brain (Elston et al., 1996, 2009, 2010a, 2010b, 2011b; Elston and Rosa 1997, 1998; Jacobs et al., 1997, 2001; Petanjek et al., 2008, 2011; Amatrudo et al., 2012; Bianchi et al., 2013). In some cortical areas, such as macaque primary visual cortex (V1), the basal dendritic trees of layer-III pyramidal cells grow to their full extent around birth, then become successively smaller through infancy, adolescence, and adulthood (Boothe et al., 1979; Elston et al., 2009, 2010a). In other cortical areas, such as inferotemporal cortex (posterior dorsal part of cytoarchitectonic area TE) and granular prefrontal cortex (gPFC; area 12; Walker, 1940) in the macaque (Elston et al., 2009, 2010a) and gPFC (Brodmann’s area [BA] 9, Brodmann, 1909) in humans (Petanjek et al., 2008; 2011), pyramidal cells grow increasingly larger basal dendritic trees from birth through adulthood; only in older age (>50), dendrites decrease their arbor size (BA10 and BA 12 in human; Jacobs et al., 1997). The numbers of spines grown and pruned from the basal dendritic trees of pyramidal cells also differ considerably among cortical areas. For example, basal dendrites of layer-III pyramidal cells in macaque V1 attain an average of 1900 spines by the age of 3.5 months (Elston et al., 2009, 2010a) — the period corresponding to peak synaptogenesis in the neuropil (Rakic et al., 1986; Bourgeois et al., 1989; Bourgeois and Rakic, 1993). A large proportion of these spines (> 75%) are then pruned, resulting in a net reduction of pyramidal cell spines between birth and adulthood (Elston et al., 2009). In contrast, layer-III cells in area TE can grow an average of 10,400 spines by 3.5 months, and those in area 12 as many as 15,900, both cases being net increases in spine number between birth and adulthood (Elston et al., 2009, 2010a).

These differences in the developmental profiles of pyramidal cells result in systematic structural differences in the adult macaque and human brain such that pyramidal cells become more spinous as brain regions transition from sensory to association to executive cortex (Elston and Rosa, 1997; Elston et al., 1999a, 2006a; Jacobs et al., 2001; see Elston, 2007, Elston & Fujita, 2014, for review). These anatomical differences have been proposed to provide a basis for specialized physiological and behavioral functions (Jacobs and Scheibel, 2002; Elston, 2007; Spruston, 2008; Amatrudo et al., 2012; Eyal et al., 2016; Mochizuki et al., 2016). However most of this research was focused on layer III. In the case of layer V, most research has been focussed on a single cortical area (visual cortex: Lund et al., 1977; Boothe et al., 1979; Takashima et al., 1980; Becker et al., 1984; motor cortex: Nakamura et al., 1985; prefrontal cortex: Mrzljak et al, 1992; Koenderink et al., 1994; Anderson et al., 1995; Koenderink and Uylings, 1995; Petanjek et al., 2008, 2011). Whether and how the developmental profiles differ across cortical areas in infragranular layers remains unclear. Furthermore, few studies have compared developmental profiles of pyramidal cells between supragranular and infragranular layers.

Here, I performed a systematic study of the basal dendritic trees of layer-V pyramidal cells in macaque V1, TE, and area 12, thus representing the hierarchical functions of primary sensory, sensory association, and executive cortex, respectively. To characterize growth, spinogenesis, and pruning throughout development and into maturity, each was assessed at four time points, the earliest being the 2nd postnatal day and the latest being at 4.5 years. I conducted experiments in the same animals in which I examined layer-III pyramidal cells in our previous studies (Elston et al., 2009, 2010a, 2010b, 2011b), thus facilitating inter-laminar comparison of the developmental process. I demonstrate that basal dendrites of layer-V cortical pyramidal cells exhibit area- and layer-specific developmental profiles.
2.2. Materials and Methods

2.2.1. Animals and care

Five male macaque monkeys (*Macaca fascicularis*) aged 2 days (D), 3 weeks (W), 3.5 months (M), and 4.5 years (Y) were used in the present study (2 monkeys at 4.5Y) (Table 2.1). The animal-experiment committee of Osaka University approved the protocols for animal care and experimentation, which were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* issued by the National Institutes of Health, USA (DHEW Publication No. (NIH) 85–23, Revised 1996, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205, USA).

The four time points were chosen because they equate to just after birth, the time including the critical period for ocular dominance shift (Horton and Hocking, 1997), the time of peak synaptogenesis (Rakic et al., 1986; Bourgeois et al., 1989; Bourgeois and Rakic, 1993), and young adulthood, respectively. These ages correspond to those studied for layer-III pyramidal cells in these same cortical areas (Elston et al., 2009, 2010a). Indeed, these data were sampled from the same animals as the layer-III data, allowing us to rule out inter-individual variation as a possible confound in inter-laminar comparisons.

<table>
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<tr>
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<td>9</td>
<td>22</td>
<td>16</td>
<td>10</td>
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<td>TE</td>
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<td>37</td>
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</tr>
<tr>
<td>12</td>
<td>27</td>
<td>35</td>
<td>19</td>
<td>20</td>
<td>9</td>
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*D, W, M, Y: postnatal days, weeks, months, and years

Total number of cells: 311

2.2.2. Intracellular dye injection in lightly fixed tissues

All methods employed in the present study were the same as those detailed in our previous studies on pyramidal cell development (Elston et al., 2009, 2010a, 2010b, 2011b). In brief, following overdose with sodium pentobarbital (> 75 mg/kg intravenously or intraperitoneally; Dainippon Sumitomo Pharma, Osaka, Japan) animals were perfused intracardially with 0.9% saline in 0.1M phosphate buffer (pH = 7.2) and then 4% paraformaldehyde in the same phosphate buffer, followed by removal of the brain. Tissue for V1 was taken from the dorsolateral region of the exposed occipital operculum, corresponding to the central 5–7 degrees of visual representation (Figure 2.1E; Daniel and Whitteridge, 1961; Oga et al., 2016). I sampled tissue for inferotemporal cortex from the middle third of the inferior temporal gyrus immediately anterior to the posterior middle temporal sulcus (TE; TEp of Seltzer and Pandya, 1978; TEpD of Yukie, 1997). Tissue for prefrontal cortex was taken from the exposed portion of the ventrolateral granular prefrontal cortex (area 12 of Walker, 1940), also known as 12vl (Preuss and Goldman-Rakic, 1991) or 47/12 (Petrides and Pandya, 2002). All tissues were taken from the right hemisphere.

The lightly perfused tissue was flat-mounted as described previously (Elston et al., 2010a) and postfixed overnight between glass slides in 4% paraformaldehyde. Tangential sections (250 μm) were
cut the following morning with the aid of a vibratome. To visualize nuclei of individual cells, I incubated the sections in $10^{-5}$ mol/L of the fluorescent dye 4,6-diamidino-2-phenylindole (DAPI; Sigma D9542, St Louis, USA) in phosphate buffer at room temperature for approximately 10 min before intracellular injection. The DAPI-labelled sections were mounted between two cellulose nitrate membrane filters (AABG02500, Millipore, Bedford, USA), the uppermost having a “window” to allow visualization of the tissue during injection. Injection was performed with a Leica micromanipulator coupled to a fixed-stage microscope (Eclipse FN1; Nikon, Tokyo, Japan) that was equipped with UV excitation (341–343 nm).

Although V1, area TE, and area 12 have area-specific laminar structures, all exhibit a distinct granular layer IV (Figure 2.1A-D). The section which contained layer V was easily identified in DAPI-labelled sections as that immediately below the neuron-dense granular layer. Layer-V pyramidal cells were injected in each slice. Care was taken to select the same region within each cortical area from which I sampled layer-III pyramidal cells in our previous studies (Elston et al., 2009, 2010a), such that cells included in the present study were located immediately below those sampled in layer III. Pyramidal cells were injected under visual guidance with continuous current (up to 100 nA), and the slices were processed for a light-stable reaction product by immunohistochemistry (Figure 2.2; see Elston and Rosa, 1997; Oga et al., 2016).

2.2.3. Morphological analysis

I selected neurons for analysis only when their basal dendritic arbors were fully contained within the tissue section (Figure 2.2A). They were reconstructed using Neurolucida software (MBF Bioscience, Williston, VT, USA) coupled to a microscope (Eclipse 80i; Nikon) that was equipped with a motorized stage (Ludl Electric Products, Hawthorne, NY, USA) and a charge-coupled device camera (CX9000; MBF Biosciences).

Dendritic field area was determined in the tangential plane as the area contained within a convex hull traced around the outermost distal dendritic terminations in reconstructions that were collapsed to yield two-dimensional images (see Figure 2.3A, inset). The cell-body area and total dendritic length were also calculated from these 2-dimensional projections for compatibility with previous studies (Elston et al., 1996, 2009, 2010a, 2010b, 2011b; Elston & Rosa, 1997, 1998). The branching profiles of dendritic trees were determined by Sholl analysis (Sholl, 1953). In this analysis, I counted intersections between the dendritic arbors and concentric circles. The circles were centered on the cell body with radii incremented at 10-µm steps (see Figure 2.3B, inset). By plotting the number of intersections against the radii, I obtained the entire Sholl profile for a neuron. The peak value of the profile was used as an index for dendritic branching complexity.
Figure 2.1: The layer structure of V1, area TE, and area 12 and the injection sites. For V1, two consecutive sections were stained by cytochrome oxidase histochemistry (A) and cresyl violet for Nissl substance (B). Sections for area TE (C) and area 12 (D) are stained only for Nissl substance. All sections were from a 2-year old cynomolgus monkey. The nomenclature by Hassler (1966) is listed left, and that by Brodmann (1909) is listed in the far left parentheses. (E) Injection sites for individual cases. Injections were made into the operculum for V1, a dorsal part of area TE immediately anterior to the posterior middle temporal sulcus (pmt) for TE, and a ventromedial prefrontal cortex, anterior to the inferior limb of the arcuate sulcus for area 12. All injections were made into layer V (asterisks). cal: calcarine, lu: lunate, io: inferior occipital, ot: occipito-temporal, amt: anterior middle temporal, st: superior temporal, arc: arcuate, pr: principal. 2D, 3W, 3.5M, 4.5Y: postnatal 2 days, 3 weeks, 3.5 months, and 4.5 years, respectively.
Spines were drawn with a Camera Lucida system at ×100 magnification (numerical aperture: 1.49; CFI Apo TIRF 100× H/1.49, Nikon), and quantified as a function of distance from the cell body to the distal tips of the dendrites. As with all of our previous studies, we selected horizontally-projecting dendrites for our calculations of spine densities to avoid trigonometric error. Spine densities were calculated per 10-μm interval along the entire length of 10 individually drawn, randomly selected, dendrites in each cortical area for each age group (Eayrs and Goodhead, 1959; Valverde, 1967). We made no distinction between different spine types (e.g., sessile and pedunculated spines; Jones and Powell, 1969). I estimated the total number of spines in the basal dendritic tree of the “average” cell in each area/age combination by combining the Sholl profile and the spine density profile. A value of the Sholl profile at a given distance from the cell body indicates the number of 10-μm dendritic segments at the distance. A value of the spine density profile at the distance indicates the number of spines along a single 10-μm dendritic segment. A product of the value of Sholl profile (the number of 10-μm segments at a given Sholl diameter) and the value of spine density profile (the number of spines per 10-μm segment) at the corresponding distance indicates the number of spines at the given distance. Finally, I obtained the total number of spines by summing up the number of spines profile across distance (Elston, 2001).

Given that shrinkage of brain tissue caused by perfusion has been estimated to be small (2.5%; see Oga et al., 2016), the measured values were not corrected for tissue shrinkage. Statistical analyses were performed with Matlab version 2016a (Mathworks, Inc., Natick, MA, USA).

2.3. Results

I sampled and analyzed 311 layer-V pyramidal cells in V1, area TE, and area 12 of five monkeys at different ages (2D, 3W, 3.5M, 4.5Y; Figure 2.2, Table 2.1). A total of 21,939 individual dendritic spines were drawn and tallied. Both basal dendrites and spines of layer-V pyramidal cells had area-specific growth profiles. The profiles were distinct from those previously reported for layer-III pyramidal cells in the same areas (Elston et al., 2009, 2010a).

2.3.1. Basal dendritic field area

Layer-V pyramidal cells in each examined area exhibited distinct developmental changes in their basal dendritic field areas. At 2D, those in V1 (mean ± standard deviation [SD]; 1.27 ± 0.59 × 10⁵ μm²), area TE (0.96 ± 0.33 × 10⁵ μm²), and area 12 (1.05 ± 0.32 × 10⁵ μm²) were not different from each other (Figure 3A; p = 0.12; Kruskal-Wallis test). At 4.5Y, the dendritic trees of pyramidal cells in area 12 were the largest (1.40 ± 0.43 × 10⁵ μm²) followed by those in area TE (0.81 ± 0.25 × 10⁵ μm²), which were larger than those in V1 (0.40 ± 0.19 × 10⁵ μm²)(p = 6.9 × 10⁻¹³; Kruskal-Wallis test; p < 10⁻⁶ for area 12 vs. area TE, and area TE vs. V1; post-hoc Mann-Whitney’s U-test). This difference resulted from two trends: a decrease in the size of the dendritic trees in V1 with age (p = 5.1 × 10⁹; Kruskal-Wallis test) and an increase in their size in area 12 over the same period (p = 7.2 × 10⁸). The dendritic trees of cells in area TE were similar in size for 2D and 4.5Y (p = 0.11; post-hoc Mann-Whitney’s U-test).
Figure 2.2: Photomicrographs of layer-V pyramidal cells individually injected in tangential sections with Lucifer Yellow and reacted for DAB product. (A) Labelled cells for areas V1, TE, and area 12 at 2 days (2D) and 4.5 years (4.5Y). Dendritic branches of pyramidal cell at 4.5Y was smaller than those at 2D in V1, did not change in area TE, and became greater at 4.5Y than at 2D in area 12. (B) Dendritic spines along a dendritic segment in the three areas at each of the four time points. Dendritic segments were poor in spines at 2D, most spinous at 3W in V1, at 3W to 3.5M in area TE and area 12, then spines were pruned toward adulthood.
The area-specific developmental changes in dendritic tree size appear to result from disappearance of V1 cells with large dendritic fields after 2W and appearance of cells with large dendritic fields in the area 12 of adults (Figure 2.4). The variance of dendritic field area significantly differed across ages in all three areas ($p = 3.2 \times 10^{-5}$, $1.5 \times 10^{-4}$, and 0.027 for V1, area TE, and area 12, respectively; Brown-Forsythe test for comparison of variance across the groups). In V1, the distribution of dendritic field area across neurons was broader at 2D ($SD = 0.59 \times 10^5 \mu m^2$) than at 3W, 3.5M, or 4.5Y ($SD = 0.75 \times 10^5 \mu m^2$, $0.34 \times 10^5 \mu m^2$, and $0.19 \times 10^5 \mu m^2$; $p < 10^{-4}$, $p = 0.018$, and $p < 10^{-4}$, for 2D vs. 3W, 3.5M, and 4.5Y, respectively; post-hoc $F$-test). V1 neurons with dendritic field area larger than $1.0 \times 10^5 \mu m^2$ were dominant at 2D, but were very few at later ages. The distribution in area TE was broader at 2D ($0.33 \times 10^5 \mu m^2$) than at 3W or 3.5M ($SD = 0.13 \times 10^5 \mu m^2$, $0.11 \times 10^5 \mu m^2$; $p < 10^{-4}$), but similar to the distribution at 4.5Y ($0.25 \times 10^5 \mu m^2$; $p = 0.25$). In area 12, neurons with large dendritic fields dominated at 4.5Y compared to the other ages (mean ± SD = $1.05 \pm 0.32 \times 10^5 \mu m^2$ at 2D, $0.98 \pm 0.32 \times 10^5 \mu m^2$ at 3W, $1.16 \pm 0.25 \times 10^5 \mu m^2$ at 3.5M, $1.40 \pm 0.43 \times 10^5 \mu m^2$ at 4.5Y; $p = 0.0013$, 0.001, and 0.045 for 2D vs. 4.5Y, 3W vs. 4.5Y, and 3.5M vs. 4.5Y, respectively; post-hoc Mann-Whitney’s $U$-test).
2.3.2. Total length of basal dendrites

Basal dendritic length was constant across ages in V1, and increased in area TE and area 12 during development (\(p = 0.058\) in V1; \(p = 1.4 \times 10^{-4}\) in area TE; \(p = 3.4 \times 10^{-5}\) in area 12; Kruskal-Wallis test among ages). At 2D, the length was similar between the three areas (mean ± SD = 3856 ± 1769 μm in V1; 3713 ± 1116 μm in area TE; 3735 ± 978 μm in area 12; \(p = 0.99\); Kruskal-Wallis test). At 3W the total length of dendrites slightly decreased in V1 (mean ± SD = 3112 ± 510 μm in V1), whereas those in area TE and area 12 increased (4743 ± 777 μm in area TE; 4874 ± 1317 μm in area 12; \(p = 0.0015\) in area TE [2D vs. 3W]; \(p = 0.0004\) in area 12 [2D vs. 3W]; post-hoc Mann-Whitney U-test), showing inter-area difference (\(p = 1.6 \times 10^{-4}\); Kruskal-Wallis test). At 4.5Y, the difference in the total dendritic length became further obvious; 2622 ± 1118 μm in V1, 4398 ± 982 μm in area TE, and 5245 ± 962 μm in area 12 (\(p = 8.7 \times 10^{-10}\); Kruskal-Wallis test). Net changes between 2D and 4.5Y were -1234 μm for V1 (\(p = 0.03\); post-hoc Mann-Whitney U-test), +685 μm for area TE (\(p = 0.068\)), and +1,510 μm for area 12 (\(p = 6.0 \times 10^{-6}\)) (Figure 2.3B).

2.3.3. Cell-body size

I plotted histograms of cell-body area to examine whether the samples in each area consisted of mixed cell populations. The distributions did not have statistically significant multiple peaks in any area/age combination (\(p > 0.055\); Hartigan’s dip test). The mean cell-body area differed across ages in all three areas (\(p = 0.0038\), 7.0 \times 10^{-9}, and 2.5 \times 10^{-9}, for V1, area TE, and area 12, respectively; Kruskal-Wallis test). In V1, the mean values at 2D (mean ± SD; 181 ± 62 μm²) and 3W (184 ± 13 μm²) were larger than the mean value at 3.5M (127 ± 31 μm²) (\(p < 0.01\); post-hoc Mann-Whitney U-test). In area TE, the mean cell-body area was largest at 4.5Y (246 ± 48 μm²) than those at 2D (166 ± 44 μm²), 3W (194 ± 37 μm²), and 3.5M (167 ± 34 μm²) (\(p < 0.01\)). In area 12, The mean cell-body area was smallest at 3.5M (142 ± 41μm²) than those at 2D (225 ± 42μm²), 3W (262 ± 58μm²), and 4.5Y (239 ± 42μm²) (\(p < 0.01\)) (Figure 2.5).

I further examined correlation between the cell-body size and the total dendritic length in each area/age group of cells. If the cell-body size is mainly determined by the metabolic demand of sustaining dendritic arbors, cells with longer total length of dendrites would have larger cell-body. In 4 out of 12 combinations (3 areas x 4 ages), the cell-body size positively correlated to the dendritic length (2D-V1, \(r_s = 0.744, p = 0.0009\); 2D-TE, \(r_s = 0.679, p = 0.043\); 3W-12, \(r_s = 0.79, p = 4.7 \times 10^{-6}\); 4.5Y-V1, \(r_s = 0.858; p = 2.3 \times 10^{-5}\); \(r_s\): Spearman’s rank correlation coefficient; \(p\)-value was Bonferroni corrected). I found no significant correlation in the other combinations. When examined for the data collapsed across all cells, there was a relatively strong correlation; the bigger the cell body, the longer the basal dendrites (\(r_s = 0.497; p = 8.7 \times 10^{-21}\)).

2.3.4. Branching patterns of basal dendrites assessed by Sholl analysis

The number of peak intersections yielded by the Sholl profile of layer-V pyramidal cell dendritic trees at 2D was greatest in area TE (mean ± SD; 25.1 ± 6.6), followed by area 12 (23.3 ± 5.9), and V1 (19.6 ± 7.2) (Figure 2.3C). Peak branching complexity was observed in the dendritic trees of pyramidal cells in V1 and area TE at 3W, with area TE complexity being considerably higher than that in V1 (38.4 ± 6.0 and 26.0 ± 5.5, respectively). In area 12, branching complexity in these cells was highest at 4.5Y (31.8 ± 6.2). In the adult, pyramidal cells in TE (29.8 ± 8.0) and area 12 (31.8 ± 6.2) had similar numbers of branch points, which were greater than the numbers of branches in V1 (24.5 ± 6.9). Statistical analysis revealed that the peak number of intersections in the Sholl profiles significantly differed across age groups in each area (\(p = 1.7 \times 10^{-3}\), \(p < 1 \times 10^{-5}\), and \(p < 1 \times 10^{-5}\), for V1, area TE, and area 12, respectively; Kruskal-Wallis test).
2.3.5. Fractal dimension

Fractal dimension—an indicator of dendritic complexity (Elston and Jelinek, 2001)—increased in all three areas \((p = 0.031, 1.2 \times 10^{-7}, \text{and } 2.3 \times 10^{-5}; \text{for } V1, \text{TE and } 12, \text{respectively}; \text{Kruskal-Wallis test})\). At 2D, the fractal dimension was similar between V1 \((1.109 \pm 0.037), \text{area } \text{TE } (1.134 \pm 0.042), \text{and area } 12 \((1.121 \pm 0.033) (p = 0.27). \text{The fractal dimension in area } \text{TE } (1.184 \pm 0.021; p = 0.0001; \text{post-hoc Mann-Whitney’s U-test [2D vs. 3W]} \text{and area } 12 \(1.158 \pm 0.030; p = 0.0001; \text{post-hoc Mann-Whitney’s U-test [2D vs. 3W]} \text{increased at 3W and difference between the areas emerged } (p = 2.2 \times 10^{-5}; \text{Kruskal-Wallis test}). \text{The fractal dimension of area } \text{TE } \text{then decreased at 3.5M } \((1.154 \pm 0.019; p = 6.9 \times 10^{-8}; \text{post-hoc Mann-Whitney’s U-test}). \text{At } 4.5Y, \text{the fractal dimension of area } \text{TE } \text{was } \((1.165 \pm 0.025) \text{and area } 12 (1.163 \pm 0.026) \text{was larger than that of } V1 (1.135 \pm 0.038) (p = 4.2 \times 10^{-4}; \text{Kruskal-Wallis test}; p = 0.0006, 0.0008, 0.83 \text{for } V1 \text{vs. TE, V1 vs. 12, and TE vs. 12, respectively}; \text{post-hoc Mann-Whitney’s U-test}). \text{Thus, the complexity of dendritic branching patterns were similar at birth between the three areas, and became different in adults with area } \text{TE} \text{and area } 12 \text{being more complex than } V1 (\text{Figure 2.3D}).

![Figure 2.4: Frequency histograms of the basal dendritic field area of layer V neurons from areas V1, TE, and 12. Arrowheads indicate the medians.](image1)

![Figure 2.5: Frequency histograms of cell-body size of layer-V neurons from areas V1, TE, and area 12. Arrowheads indicate the medians.](image2)

2.3.6. Spine densities of the basal dendrites

At 2D, peak spine density (spines/μm) along dendritic segments in area 12 \((\text{mean } \pm \text{SD}; 1.07 \pm 0.39) \text{was greater than that observed in V1 or area } \text{TE } (0.77 \pm 0.31 \text{and } 0.70 \pm 0.16, \text{respectively}) (\text{Figure 2.6}; \text{see also Figure 2.2B}). \text{The greatest peak spine density in V1 was observed at 3W (2.42 \pm 0.42), which was lower at each successive time point (3.5M: 1.03 \pm 0.34; 4.5Y: 0.65 \pm 0.30). \text{The greatest peak spine densities in TE and area 12 were observed at 3W (1.85 \pm 0.30 \text{and } 1.86 \pm 0.41, \text{respectively}) and 3.5M (2.04 \pm 0.41 \text{and } 1.88 \pm 0.24, \text{respectively), being approximately 30% higher than those}
observed at 4.5Y (1.21 ± 0.33 and 1.25 ± 0.31, respectively) (Figure 2.6). At 4.5Y, the peak densities in TE and area 12 were about 1.9 times higher than that in V1 (0.65 ± 0.30). A statistical test revealed these differences in spine density were significant across ages ($p < 10^{-5}$; for V1, TE, and area 12; Kruskal-Wallis test). Thus, all three areas attained peaks in spine density as early as 3W. At 3.5M, density had already substantially decreased in V1, whereas it was still at peak levels in TE and area 12.

Figure 2.6: Spine density plots for the basal dendrites of layer-V pyramidal cells, sampled from areas V1, TE, and area 12 at 2 days (2D), 3.5 months (3.5M), and 4.5 years (4.5Y). Each profile indicates dendritic spine density along the dendritic extent. Shaded areas indicate standard deviation.

Estimates of the total number of spines in the basal dendritic tree of the "average" pyramidal cell revealed striking differences in postnatal changes between the three areas. At 2D, cells in area 12 were considerably more spinous (2198) than those in V1 (1395) or area TE (1408) (Figure 2.7). By 3W, cells in V1, area TE and area 12 had 2–3 times more spines, with levels evening out across areas (4840, 5167, 5214, respectively). At 3.5M, the numbers of spines in area 12 (4943) and area TE (5825) had increased, while that in V1 was reduced by about 60% (1742). By 4.5Y, the numbers of spines in the dendritic tree of the “average cell” in area 12 (3287) and TE (3076) were approximately 30% less, while in V1 the number had decreased by 40% (1000) (Figure 2.7). Thus, the total number of spines in the basal dendrites of V1 pyramidal cells quickly decreased after the peak at 3W, and continued to decrease into the 5th year of life, resulting in adults having fewer spines than newborn monkeys. In contrast, area TE and area 12 exhibited broader peaks that spanned from 3W to 3.5M, with adults having more spines than newborns.
Chapter 2. Spinogenesis and pruning in Layer-V

2.4. Discussion

In the present investigation, I studied the basal dendritic tree morphology of layer-V pyramidal cells in V1, cytoarchitectonic area TE, and Brodmann’s area 12 in macaque monkeys that ranged in age from 2 days to 4.5 years. The main aim of the study was to find differences, if they exist, in the developmental profiles of layer-V pyramidal cells across these cortical areas. The profiles were indeed area-specific. Layer-V pyramidal cells in V1 retracted basal dendritic arbors postnatally, whereas those in area TE remained constant in size, and those in area 12 became larger, increasing the size of their dendritic field areas (Figures 2.3, 2.4, 2.8A). Layer-V pyramidal cells in V1 exhibited peak spine density and the greatest number of spines at 3W, whereas area TE and area 12 maintained the greatest density and numbers of spines over a longer period, spanning 3W and 3.5M (Figures 2.6, 2.7). This early maturation distinguishes layer V from layer III in which pyramidal cells in all three areas concurrently reach peak density and total number of spines at 3.5M (Elston et al., 2009; 2010a).

2.4.1. Area-specific postnatal changes in pyramidal cell morphology

The results revealed relative differences in the development of pyramidal cell dendritic trees among the three cortical areas. Specifically, basal dendritic trees of cells in V1 were largest at 2D and continued decreasing in size into adulthood, those in TE were similar in size at 2D and 4.5Y, and those in area 12 increased in size from birth into adulthood (Figure 2.3). Peak spine density was greatest at 3W in V1, and lasted from 3W to 3.5M in area TE and area 12 (Figure 2.6). Estimates of the total number of spines in the dendritic trees revealed that pyramidal cells in V1 attained their greatest number of spines at 3W, and these were subsequently pruned by 3.5M and again by 4.5Y (Figure 2.7). Cells in adult V1 contained less than 20% the number of spines observed at the 3-week-old peak. Cells in area TE and area 12 attained their greatest numbers of spines at 3.5M, and those in adults contained approximately 40% (TE) and 50% (area 12) of their peak numbers. Of note, cells in V1 lose more spines than they grow following the onset of visual experience, whereas those in area TE and area 12 grow more spines than they lose during the same period (Figure 2.7; compare the colored horizontal bars that denote adult levels for the three areas at 2D). These data provide further evidence for regional variation in pyramidal cell development across different cortical areas, including infragranular cells.

While in general peaks in synaptogenesis may occur approximately 3.5 months after birth across cortical areas and layers (Bourgeois et al., 1994; Bourgeois and Rakic, 1993; Rakic et al., 1986), the

![Figure 2.7: Total number of dendritic spines in the basal dendritic tree of the “average” cell in each cortical area and age. Horizontal bars with different colors indicate the adult levels for areas V1, TE, and area 12.](image)
basal dendritic tree spine count of layer-V cells in V1 was greatest at 3W, followed by a net decrease of approximately 60% by 3.5M (Figure 2.7). In area 12, dendritic trees continued to grow from 2D into adulthood. These larger and more branched dendritic trees are replete with spines, which presumably accommodate functioning asymmetrical synapses (e.g., Arellano et al., 2007), suggesting that additional functional synapses are grown beyond 3.5M into adulthood.

In the present analysis, I did not consider the heterogeneity of pyramidal cells in layer V. Layer V is composed of two sublayers, VA and VB (Brodmann, 1909). Cell density is greater in VA than VB (Figure 2.1B-D), and cell bodies are larger on average in layer VB than in layer VA. I did not distinguish these sublayers. Our samples from each area and age might thus include pyramidal cells with different ratios of VA and VB neurons. In addition to the sublayers, layer V contains subtypes of pyramidal cells that differ in their projection targets (i.e., cortical vs. subcortical) (O’Leary and Koester, 1993). Subcortically projecting neurons have larger cell bodies and thicker and more extensive apical dendrites than cortically projecting neurons (see Feldman, 1984 for review; Kim et al., 2015). Although the differences were not statistically significant, an inspection of cell body-size distribution in V1 suggests that two subpopulations, large cells and smaller cells, might exist in our V1 dataset (Figure 2.5). The data at 2D and 4.5Y may include both subpopulations, whereas the data at 3W and 3.5M might include only one of them. Despite these caveats, I believe that the decrease in dendritic field area in V1 was genuine because the data at 2D and 4.5Y include both subpopulations.

In addition to dendrites, pyramidal cell axons also exhibit area-specific morphology and developmental changes. The size, spacing, and maximal extent of the terminal patches belonging to intrinsic horizontal axons of layer-III pyramidal cells are greater in area TE than in V1 (Fujita and Fujita, 1996; Tamigawa et al., 2005). These characteristics are already evident one week after birth, and are refined by postnatal development into the adult-type phenotype (Wang et al., 2016). The number and distribution of patches, but not the inter-patch distance, gradually decrease with age in V1, suggesting that the furthest patches in infants are later pruned. In contrast, this change in the number and extent of patches does not occur in area TE. Future studies are required to determine how genetic and epigenetic mechanisms interact to produce regional specialization of dendrites and axons in the primate brain (Whitford et al., 2002; Malyshevskaya et al., 2013; Sasaki et al., 2014a, 2014b; Bakken et al., 2016).

2.4.2. Layer-specific development of pyramidal cells: comparison between layer V and layer III

Previously, I showed that basal dendritic field area of layer-III pyramidal cells in area TE and area 12 increased with age, whereas those of V1 cells decreased (Figure 2.8B). In contrast, while the trends in layer V were similar to those in layer III for V1 and area 12, dendritic field area remained relatively unchanged over time in area TE (Figure 2.8A).

Overproduction and pruning of spines was a common feature between the two layers, but the time course was different. In layer III, spine density peaked at 3.5M in all three areas (Elston et al., 2009), whereas it peaked earlier in layer V (3W in V1 and from 3W to 3.5M in area TE and area 12; Figure 2.6). Inter-area differences in spine density became obvious only after 7 months in layer III. In contrast, spine density in layer-V cells exhibited inter-area differences throughout development. Thus, within each area, postnatal pyramidal cell development differs between layer III and layer V. However, the overall changes in the total number of spines were similar between layer III and layer V in that pyramidal cells in both layers showed increasing numbers of dendritic spines with age in area TE and area 12 and decreasing numbers in V1 (Elston et al., 2009 for layer III; Figure 2.7 for layer V).
Figure 2.8: Schematic illustration showing area-specific and layer-specific developmental changes of pyramidal cell dendrites. The cells are drawn proportional in size to the mean dendritic field areas of corresponding area/age groups. Layer-V pyramidal cells in V1 retract basal dendritic arbors postnatally, whereas those in area TE remain constant in size, and those in area 12 become larger, increasing the size of their dendritic field areas (A). Layer-III basal dendritic field areas of pyramidal cells in area TE and area 12 increase with age, whereas those of V1 cells decrease (B). The scheme for layer V is based on the current results, and that for layer III is based on Elston et al. (2009, 2010a).

2.4.3. Inter-individual differences in pyramidal cell morphology

In general, the variation in age/gender/area/layer/topography matched pyramidal cell structure among individuals is markedly less than differences observed between cortical areas within an individual or within a given cortical area at different developmental ages. Data sampled from a specific cortical area/layer in a number of different individuals have been reported for each individual in the galago (Elston et al., 2005), the marmoset (cf. Elston et al., 1996 & Elston et al., 1999b), the South American Agouti (Elston et al., 2006b) and macaque (Oga et al., 2016), revealing highly conserved structure in age/gender/area/layer/topography matched pyramidal cells among individuals. The exception, thus far, is the granular prefrontal cortex where studies in the baboon, vervet monkey and macaque monkey reveal inter-individual variation in pyramidal cell structure among age/gender/area/layer/topography matched pyramidal cell structure not observed in any other cortical region (Elston et al., 2011a – in particular, see Figure 7).

In the current dataset, our samples at 4.5Y were obtained from two monkeys. I evaluated an inter-individual difference by comparing the total dendritic length, a factor that strongly influences the total number of spines. There was a difference between the two monkeys in V1, whereas no difference was found in area TE and area 12. The ratio of the total dendritic length between the two monkeys were 1.98 in V1 (1,902 μm vs. 3,775 μm; p = 5.5 × 10^-5; Mann-Whitney’s U-test), 1.14 in area TE (4,144 μm vs. 4,743 μm; p = 0.16), and 1.08 in area 12 (5,368 μm vs. 4,972 μm; p = 0.21). The inter-individual difference in V1 may be caused by a difference in cortical depth sampled (e.g. sub-layer 5A or 5B), as suggested by the bimodal distribution of cell-body size (see Figure 5). When compared between the two monkeys, the cell body size was different in V1 (MF1, 92.6 ± 17.9 μm^2, n = 16; CI15, 216.7 ± 45.5 μm^2, n = 10; p = 2.8 × 10^-5), but similar in area TE (MF1, 260.4 ± 43.9 μm^2, n = 15; CI15, 225.3 ± 47.5 μm^2, n = 11; p = 0.049) and area 12 (MF1, 241.4 ± 45.2 μm^2, n = 20; CI15, 234.5 ± 35.3 μm^2, n = 9; p = 0.80). In contrast to the individual differences of the total dendritic length, the largest change
over the postnatal development was 1.47 in V1 (3,856 [2D] vs. 2,622 [4.5Y]), 1.34 in area TE (4,743 [3W] vs. 3,713 [2D]), 1.45 in area 12 (3,735 [2D] vs. 5,245 [4.5Y]), showing that the developmental change in area TE and area 12 was larger than the individual difference.

### 2.4.4. Comparative development of pyramidal cells in human and other primate species

A continuous decrease in the size of the basal dendritic field in layer-III and layer-V pyramidal cells during development is a striking feature of the primary sensory cortices in cynomolgus monkeys (layer III in V1, Elston et al., 2010a; layer V in V1, current study; layer III in primary auditory cortex, Elston et al., 2010b). In V1 of human and other non-human primate species, this decrease is preceded by an initial growth. The period at which the decrease begins varies between species; from 3W to 15W in layer IIIB in southern pig-tailed macaques (Boothe et al., 1979), and 12–24 months in human layer V (Becker et al., 1984). In a New World primate (marmoset), layer III pyramidal cells in V1 do not exhibit postnatal decrease in the size of their dendritic arbors (Oga et al., 2013).

Pyramidal cells in prefrontal cortex (both ventrolateral and dorsolateral) grow their dendrites from infancy to adulthood. This continuous growth is a common feature in marmosets (layer III in area 12; Oga et al., 2013), cynomolgus monkeys (layer III and V in area 12; Elston & Fujita, 2014 and the current study) and humans (layer III in BA9 and BA46: Koenderink et al., 1994; layer V in BA9 and BA46: Koenderink & Uylings, 1995; layers III and V in BA9: Petanjek et al., 2008).

At birth, dendritic branches of pyramidal cells in human cerebral cortex are on average longer in layer V than in layer III (Becker et al., 1984 for V1; Mrzljak et al., 1992 for BA9 and BA46; Petanjek et al., 2008 for BA9). Pyramidal cells in layer V are born earlier, and might start to grow dendrites earlier than cells in layer III (Mrzljak et al., 1992). Dendritic field area in cynomolgus monkeys at 2D was also larger in layer V than in layer III in each area (Figure 8A, B; comparison between current study and Elston et al., 2009, 2010a).

Patanjek and colleagues (2008) reported that layer III pyramidal cells in human BA9 undergo development in two stages. As discussed above, dendrites are less developed in length at birth in layer III than in layer V. Within one month, dendrites in layer III rapidly grow and catch up to the size of dendrites in layer V. After this initial growth, layer III neurons maintain their size during the 3 to 16 months after birth (steady period). At this point (around age 2.5 years), the second growth stage begins. In the macaque monkey, area 12 pyramidal cells in both layers III and V maintain the same dendritic field size between 2D and 3W, but then show rapid dendritic growth from 3W to 3.5M (Elston et al., 2009; current study). The period from 2D to 3W in monkeys may correspond to the steady period observed in human BA9. The rapid growth of layer-III dendrites is accompanied by peak synaptogenesis and spinogenesis in both human (2 to 2.5 years; Huttenlocher and Dabholkar, 1997 for middle frontal gyrus; Petanjek et al., 2011 for BA9) and macaque (2 to 4 months; Bourgeois et al., 1994; Bourgeois and Rakic, 1993; Rakic et al., 1986; Elston et al., 2009).

### 2.4.5. Changes of pyramidal cell dendrites and spines in older age

Dendritic length and the number of spines gradually decrease in older age. Layer III pyramidal cells in human BA10 and BA18 had shorter basal dendrites and a smaller number of dendritic spines in an older group (> 50-year old) than in a younger group (≤ 50-year old) (Jacobs et al., 1997). Spines on basal and oblique dendrites of both layer III and V pyramidal cells in human BA9 also continuously decrease after puberty (Patanjek et al., 2011). In macaque monkeys, the total number of spines in basal dendrites decline from 3,076 at 4.5Y (this study) to 2,112 at 16Y (Elston and Rosa, 2000) in layer V pyramidal cells of area TE, with the magnitude of change roughly corresponding to one S.D.
2.5. Conclusion

The data sampled from layer V provide further evidence that pyramidal cells in different cortical areas are characterized by different growth profiles, and expands the findings in previous reports with regard to regional specialization in layer-III cells. Further, when compared directly with pyramidal cells sampled from layer III of the same animals, the current data reveal that within a given cortical area, pyramidal cells in infragranular layers may have different growth profiles compared with those immediately above in supragranular layers. These different growth profiles result in fundamentally different dendritic trees among different cortical areas and impact dendritic function and neuronal circuits throughout the cortical depth. Further investigation into regional and laminar specializations in developing and mature pyramidal cells, and the functional implications of the structural specialization in human and monkey, will likely yield fruitful insights into human and non-human behavior (see Elston 2007; Spruston, 2008; DeFelipe 2011; Elston and Fujita, 2014 for reviews).
Chapter 2. Spinogenesis and pruning in Layer-V

2.5. References


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Chapter 2. Spinogenesis and pruning in Layer-V


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Chapter 2. Spinogenesis and pruning in Layer-V


Chapter 3. Basal dendrites of layer-III pyramidal neurons do not scale with changes in cortical magnification factor in macaque primary visual cortex: dendritic homogeneity within area

In this chapter, I examined dendritic variation within a cortical area.

Cortical pyramidal neurons in layer III have a wider dendritic field in higher cortical areas. For example, macaque pyramidal cells in the prefrontal cortex have greater dendritic branches than in visual association cortex, followed by lower visual areas (the fourth visual area, the secondary visual area [V2], and the primary visual area [V1]). The fact raises a question whether the differences arise out of a location in the anteroposterior axis. In the visual pathway, higher cortical areas are placed in the anterior part of lower areas, that is, V1 is located in the posterior part of the brain and V2 are in front of V1. If anteroposterior location reflects the dendritic branch size, the trend can be seen within an area. V1 is the best candidate for asking the question because V1 occupies the widest region in cortical areas in macaque monkeys.

Neurons in the mammalian primary visual cortex (V1) are systematically arranged across the cortical surface according to the location of their receptive fields, forming a visuotopic (or retinotopic) map. Within this map, the foveal visual field is represented by a large cortical surface area, with increasingly peripheral visual fields gradually occupying smaller cortical areas. Although cellular organization in the retina, such as the spatial distribution of ganglion cells, can partially account for the eccentricity-dependent differences in the size of cortical representation, whether morphological differences exist across V1 neurons representing different eccentricities is unclear. In particular, morphological differences in dendritic field diameter might contribute to the magnified representation of the central visual field. Here, I addressed this question by measuring the basal dendritic arbors of pyramidal neurons of layer-IIIC and adjoining layer III (in the Hassler's nomenclature) in macaque V1. I labeled layer-III pyramidal neurons at various retinotopic positions in V1 by injecting lightly fixed brain tissue with intracellular dye, and then compared dendritic morphology across regions in the retinotopic map representing 0–20° of eccentricity. The dendritic field area, total dendritic length, number of principal dendrites, branching complexity, spine density, and total number of spines were all consistent across different retinotopic regions of V1. These results indicate that dendrites in layer-III pyramidal neurons are relatively homogeneous according to these morphometric parameters irrespective of their locations in this portion of the retinotopic map. The homogeneity of dendritic morphology in these neurons suggests that the emphasis of central visual field representation is not attributable to changes in the basal dendritic arbors of pyramidal neurons in layer III, but is likely the result of successive processes earlier in the retino-geniculo-striate pathway.

Part of this chapter has been published in the following article.

Chapter 3. Dendritic homogeneity in V1

3.1. Introduction

In the mammalian primary visual cortex (V1), visual information from the left and right visual fields is processed in the contralateral hemisphere. Across the cortical sheet of each hemisphere, neurons are systematically arranged according to the location of their receptive field (RF). Nearby neurons in the cortex respond to visual inputs originating from nearby locations in the visual field (i.e., in the retina) forming a visuotopic (retinotopic) map. The retinotopic map was independently discovered by Inouye (1909) (translated in Glickstein & Fahle, 2000) and Holmes and Lister (1916) by analyzing the spatial relationship between visual field deficits and the gunshot path through the skull of wounded soldiers. As these pioneering studies already noticed and later studies on human and animals detailed, the foveal visual field is represented by a large cortical surface area, while gradually smaller areas are allocated to more peripheral visual fields (Daniel & Whitteridge, 1961; Gattass et al., 1981, 1987; Tootell et al., 1982; Van Essen et al., 1984; Fritsches & Rosa, 1996).

How much cortex is devoted to a given visual field can be quantified by the cortical magnification factor, which is defined as cortical surface area divided by the size of visual field represented in it (mm²/deg²; Talbot & Marshall, 1941; Daniel & Whitteridge, 1961). In human V1, the magnification factor has been estimated to be 16 mm²/deg² at 2° and 0.25 mm²/deg² at 25° (Cowey & Rolls, 1974), and in squirrel monkeys, it was shown to be 54.4 mm²/deg² for the foveal field (0°), 31.5 mm²/deg² at 0.5°, 9.6 mm²/deg² at 2°, 0.10 mm²/deg² at 20° and 0.01 mm²/deg² at 50° (Adams & Horton, 2003). The gradual decrease in cortical magnification factor as visual fields move toward the periphery can be partially explained by the fovea-centric distribution pattern of ganglion cells in the retina. For example, in squirrel monkeys the density of retinal ganglion cells (RGCs) in the foveal region (0–1°) is 5 times higher than in the near periphery (2–4°) and 235 times higher than in the far periphery (50–71°) (Adams & Horton, 2003). If the amount of divergent and convergent retinal projections to V1 is constant across visual eccentricities, the cortical magnification factor should decrease from center to periphery at the same rate as the RGC density. Assuming this linear relationship, the cortical magnification factor in the squirrel monkey fovea (at 0.5°) should be about 235 times larger than that in the periphery (at 50°). However, it is actually 3,150 times larger (31.5/0.01), which is much greater than expected. Thus, changes in cortical magnification factor roughly parallel those in RGC density, but full explanation needs further neuronal mechanism that amplifies the emphasis of the central visual field (Myerson et al. 1977; Schein & de Monasterio, 1987).

Chaplin and his colleagues (2013) suggest that the retina–lateral geniculate nucleus (LGN)–V1 projections are more divergent for central visual fields than for peripheral visual fields. They estimated the area activated by a single point of light, point image size (Capuano & McIlwain, 1981), of V1 in marmoset monkeys by mapping the retinotopy with electrophysiological techniques accurately on distortion-corrected cortical surface and multiplying cortical magnification factor with receptive field size. The point image size was larger in central vision than in peripheral vision, indicating that projection from RGCs to V1 is more divergent in the central visual field. The divergent projections from central visual field can occur at several levels of the visual pathway; the projection from retina to LGN, the projection from LGN to the input layer of V1 (i.e., layer IV), or intracortical projections within V1 (e.g., layer IV to layer III). The divergence of projections can be quantified by calculating the ratio of magnification factors at two successive stages (e.g., the V1 magnification factor divided by the LGN magnification factor). These ratios have been obtained experimentally, with that for the LGN vs. retina being 3.5 times higher in central vision than in peripheral vision (Connolly & Van Essen, 1984; Adams & Horton, 2003). Similarly, the ratio of V1 vs. LGN is 6 times higher for central visual fields than for those in the periphery (Adams & Horton, 2003). These findings indicate that divergent projections underlying the large cortical representation of central visual fields occurs both at the levels of the LGN and V1.

Two potential neural mechanisms can explain these divergent projections. In one scenario, afferent axon terminals of neurons that represent the central visual field branch more extensively and make subsequent synaptic connections with target neurons in a wider area than do those representing peripheral visual fields (Figure 3.1A, afferent specialization hypothesis). In the other scenario, the
amount of axon branching is constant across eccentricities, but neurons representing the central visual field have longer dendritic arbors with more branching than those representing the peripheral visual fields (Figure 3.1B, dendrite specialization hypothesis). This would allow neurons distributed over a wider cortical surface share the same afferent inputs in the foveal region than in the peripheral region. Both these scenarios can be implemented in the LGN and in V1 (layer IV and layer II-III). Florence and Casagrande (1987) demonstrated that the first scenario is implemented in layer IV of V1; the geniculo-striate axons of neurons carrying information from the central visual field branch more extensively than those from the peripheral visual fields. No studies have yet tested either scenario in layer III of V1. In the present study, I tested the second scenario by examining dendritic morphology of layer III pyramidal neurons at various eccentricities in macaque V1. I used intracellular dye injection to examine whether dendritic morphology of V1 neurons differed across a wide region in the retinotopic map representing 0-20° visual eccentricity. Our results showed that dendritic extent, branching complexity, spine density, and total number of spines were similar across layer-III neurons, regardless of their eccentricity. This indicates that the geometrical sampling of these neurons is uniform in this portion of the retinotopic map. I suggest that the large cortical area devoted to central vision is not achieved by the dendritic morphology in layer-III pyramidal neurons of V1, but rather by processes at the preceding sites along the retino-geniculo-striate pathway.

Figure 3.1: Schematic illustrations of possible bases for high foveal representation in the cortex. (A) The afferent specialization hypothesis. Axons of projecting neurons cover a wider area in central vision than in peripheral vision. (B) The dendrite specialization hypothesis. Dendritic arbors of target neurons (in this case, in V1) cover a wider area in central vision than in peripheral vision. In both cases, each neuron sends its signals to a larger number of V1 neurons (twice the number of neurons than periphery) if they represent central vision than if they represent peripheral vision. As represented in gray shaded areas in V1, the greater magnification in central vision is implemented in both cases. Our results demonstrated that the morphology of layer-III pyramidal neurons in V1 was uniform across the retinotopic map, and that dendritic specialization did not occur in these cells.
3.2. Materials and Methods

3.2.1. Animals and care

Three male cynomolgus macaques (*Macaca fascicularis*) aged 4.5–7.5 years were used in the experiments. All were raised at Shiga Medical School (Otsu, Shiga, Japan; Table 1). The animal experiment committee of Osaka University (Suita, Osaka, Japan) approved the protocols for animal care and experimentation, which were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (DHEW Publication No. (NIH) 85–23, Revised 1996, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205, USA).

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3.2.2. Electrophysiological mapping of retinotopic organization

One monkey (CI14) was used for mapping the retinotopic organization of V1 electrophysiologically before intracellular dye injection. The animal underwent aseptic surgery for the placement of a plastic post on the skull for head restraint. The monkey was first premedicated with atropine sulfate (0.1 mg/kg administered intramuscularly [i.m.]; Mitsubishi Tanabe Pharma, Osaka, Japan) to reduce salivation and to promote sedation during surgery. It was then sedated with ketamine HCl (Ketalar®, 25 mg/kg administered i.m.; Sankyo, Tokyo, Japan). Surgical anesthesia was accomplished with isoflurane (Forane®, 0.5%–2% in a mixture of 70% nitrous oxide and 30% oxygen; Abbott Japan, Tokyo, Japan). The local anesthetic lidocaine (AstraZeneca, London, UK) was applied to pressure points or incision sites before mounting the monkey in the stereotaxic instrument or making incisions. After exposing the top of the skull with a scalpel blade, the head post was fixed with acrylic...
resin to four stainless steel bolts inserted into the skull. Throughout the surgery, heart rate, exhaled carbon dioxide (ECO₂), and peripheral oxygen saturation (SpO₂) were continuously monitored, and body temperature was maintained near 37°C with a heating pad. After surgery, the monkey was treated with the antibiotic cefotiam hydrochloride (Pansporin®, 8 mg/kg administered i.m.; Takeda Pharmaceutical, Osaka, Japan), the analgesic ketoprofen (Menamin®, 0.8 mg/kg administered i.m.; Sanofi Aventis, Tokyo, Japan), and the corticosteroid dexamethasone sodium phosphate (Decadron®, 0.1 mg/kg administered i.m.; Banyu Pharmaceutical, Tokyo, Japan).

After a recovery period of one week, I examined eyes with a keratometer (KR-7100, Topcon, Tokyo, Japan) to select appropriate contact lenses that allowed images at a distance of 114 cm to be focused on the retina. Photographs of the retinal fundus were taken with a retinal camera (TRC-50X, Topcon) to determine the positions of the optic disc and area centralis (see Wang et al., 2002, for further details).

After another week, I performed an electrophysiological experiment to determine the retinotopic map in the right hemisphere of V1. The monkey was initially anesthetized as described above. Then, during the recording session, isoflurane and nitrous oxide were removed, and the monkey was infused with fentanyl citrate (Fentanes®, Daiichi-Sankyo, Tokyo, Japan; 0.035 mg/kg/h), and immobilized with pancuronium bromide (Miobloc®, Organon Japan, Osaka, Japan; 0.02 mg/kg/h) (Popilskis & Kohn 1997). The lactated Ringer solution with 5% glucose (Terumo, Tokyo, Japan) for infusion (10 ml/h) contained atropine sulfate (0.01 mg/kg/h), an antibiotic (Pentcilin®, Toyama Chemical, Toyama, Japan; 0.04 mg/kg/h, intravenous [i.v.]), and riboflavin (Bisulase®, Toa Eiyo, Tokyo, Japan; 0.8 mg/kg/h, i.v.). The pupils were dilated and the lenses were relaxed by applying 0.5% tropicamide-0.5% phenylephrine hydrochloride (Mydrin®-P, Santen, Osaka, Japan). The corneas were covered with contact lenses of appropriate refractive power and curvature with an artificial pupil (diameter, 3 mm) to focus on a tangent screen placed 114 cm away. The center of the screen was aligned to the projection of the fovea.

I extracellularly recorded single-cell activity using tungsten electrodes (impedance: 0.5–1.2 MΩ at 1 kHz; FHC, Bowdoin, ME, USA). A motorized micromanipulator (PC-5N; Narishige, Tokyo, Japan) was used to control the electrodes. After isolating action potentials from a single neuron, I mapped the position and size of its RF using light-bar stimuli. Bar stimuli of varying lengths and orientation were projected from a retinoscope onto a white screen placed 114 cm from the eyes in a dark environment. For each penetration, I recorded three cells at an interval of 300 μm (Figure 3.2A–C). After completing the physiological recording, the monkey was deeply anesthetized with sodium pentobarbital, perfused with fixative and buffered saline, and subjected to dye injection experiment (see the next section for details).
Chapter 3. Dendritic homogeneity in V1

Figure 3.2: Retinotopic mapping of the primary visual cortex (V1) in monkey CI14. Extracellular single-unit recording revealed the retinotopy of V1 in CI14. (A) Asterisks indicate the points penetrated on the brain surface. (B) Flat-mounted tissue for dye injection. Asterisks indicate the tracks on the slice tissue. (C) The size and location of the receptive field (RF) in the visual field. The three overlapping rectangles at each penetration (P1-P3), indicate RFs recorded from different depths along the penetration. lu: lunate sulcus. ec: ectocalcarine sulcus. (D, E) Schematic drawings that show how the V1 tissue was unfolded and flattened. Gray lines indicate approximate eccentricity lines on V1.

3.2.3. Intracellular dye injection

The intracellular dye injection techniques and immunohistochemical procedures have been described in detail elsewhere (Elston & Rosa 1997; Elston et al., 2010). Briefly, animals were overdosed with sodium pentobarbital (Nembutal®, >75 mg/kg, administered intravenously; Dainippon Sumitomo Pharma, Osaka, Japan) and perfused intracardially with 0.1 M potassium phosphate buffer saline (PBS, pH 7.2) and 4% paraformaldehyde (Merck, Kenilworth, NJ, USA). Tissue blocks were taken from the occipital lobe including V1. I removed white matter from the blocks, and unfolded the remaining gray matter (see Fig. 3.2D–E). The unfolded gray matter was flattened by sandwiching it between two glass-slides, and was postfixed overnight in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (PB).
I cut the flattened gray matter into sections tangentially to the cortical surface with the aid of a vibratome (Vibratome® Series 1500; The Vibratome Company, St. Louis, MO, USA). I then cut the blocks alternately into 250-µm and 50-µm thicknesses. The 250-µm thick sections were used for fluorescent dye injection. I used the 50-µm sections for staining Nissl substance with Cresyl violet to visualize neuronal cell-bodies and determine the cortical layers. For the 250-µm thick sections for dye injection I first stained cellular nuclei by soaking them in a solution containing $10^{-5}$ M of 4,6-diamidino-2-phenylindole (DAPI, D9542; Sigma-Aldrich, St. Louis, MO, USA) in PB at room temperature for 3 min. The section was then placed between Millipore filters (AABBG02500; EMD Millipore, Billerica, MA, USA). The Millipore filter above the section had a 6-mm in diameter circular hole to allow microelectrode access to the section. The section was then immersed in PBS and mounted in a custom-made plastic dish on a fixed-stage microscope (Eclipse FN1; Nikon, Tokyo, Japan), and was illuminated with light for ultraviolet–blue excitation (380–420 nm). Under visual guidance, I impaled DAPI-labeled neurons and applied a negative voltage to the microelectrode (up to 20 nA; Dual Microiontophoresis Current Generator, World Precision Instruments, Inc., FL, USA) to generate a continuous current for injecting Lucifer Yellow (Lucifer Yellow CH dilithium salt, L-0259, dissolved in 0.05 M Tris buffer; Sigma-Aldrich).

I focused our analysis on pyramidal neurons in the lower part of layer III. For reasons outlined elsewhere (Casagrande & Kaas 1994; Elston & Rosa 1998), I used the nomenclature of Hassler (1966) for the cortical layer. Hassler’s layer III includes layers IVA and IVB in addition to layer III as characterized by Brodmann (1909) (Fig. 3A, B; Balaram et al. 2014). I were able to unambiguously distinguish between layer III and layer IV in the DAPI-labeled sections under fluorescent microscope because neurons are denser and smaller in layer IV than in layer III (See Figure 3 of Elston & Rosa, 1997). I aimed electrodes to neurons with a round nucleus (stained with DAPI) immediately above the granular cell layer. All these neurons turned out to be pyramidal neurons; they possessed a stump of an unambiguous apical dendrite, and exhibited dendritic spines. Our sample may thus include a mixed population of pyramidal neurons from layers IVA, IVB, and IIIB of Brodmann, but not non-pyramidal neurons such as spiny stellate cells and inhibitory interneurons (see asterisks in Fig. 3.3).

After completing injections in a sufficient number of neurons, we processed the section to generate a light-stable reaction product (Elston & Rosa, 1997). The sections were immersed in a solution (2% bovine serum Albumin [A3425; Sigma-Aldrich], 1% Triton X-100 [Sigma-Aldrich], 0.1% sodium azide, and 5% sucrose in PB) containing 0.6 µg/mL biotinylated anti-Lucifer yellow (A-5751; Invitrogen, Carlsbad, CA, USA) for 4–11 days at room temperature to let the antibody infiltrate into thick tissues (250 µm). They were then washed three times for 10 min each in PB, incubated in streptavidin–biotinylated horseradish peroxidase complex (1:100, RPN1051; GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 2 h, and washed three times for 10 min each in PB. I put the tissue into 1% hydrogen peroxide in 0.1M PB for 5 min. This process helped keep the background staining minimum. After washing the sections three times for 10 min each in PB, they were incubated in 0.5% 3,3’-diaminobenzidine tetra-hydrochloride (DAB, D5637, 1:200 in PB; Sigma-Aldrich) for 10 min at room temperature. The sections were then reacted in a solution containing 1% hydrogen peroxide and 0.5% DAB in PB. This method yields a robust, light-stable reaction product.
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3.2.4. Morphological analysis

I selected neurons for analysis only when their basal dendritic arbors were fully contained within the tissue section. They were reconstructed with Neurulcida software (MBF Bioscience, Williston, VT, USA) coupled to a microscope (Eclipse 80i; Nikon) that was equipped with a motorized stage (Ludl Electric Products, Hawthorne, NY, USA) and a charge-coupled device camera (CCD; CX9000; MBF Biosciences).

The dendritic field area was determined in the tangential plane as the area contained within a convex hull traced around the outermost distal dendritic terminations in reconstructions collapsed to yield two-dimensional (2D) images (see Figure 3.7A inset). The cell-body area and total dendritic length were also calculated from these 2D projections for compatibility with previous studies on primate V1 (Elston et al., 1996, 2009, 2010; Elston & Rosa, 1997, 1998). The branching profiles of dendritic trees were determined by Sholl analyses (Sholl, 1953). In this analysis, I counted intersections between the dendritic arbor and concentric circles. The circles had their center on the cell body with varying radii incremented at 10-µm step. By plotting the number of intersections against the radii, I obtained the entire Sholl profile for a neuron. The profile visualizes branching-point location and dendritic extent.

Dendritic spines are loci for excitatory synapses (Gray, 1959; DeFelipe et al., 1988; Arellano et al., 2007). I determined the profile of spine densities along a dendrite by counting the number of spines per 10-µm segment using a 100× oil-immersion objective with correction collars (numeric aperture: 1.49; CFI Apo TIRF 100× H/1.49, Nikon) (Valverde 1967). The objective lens with correction collars can focus deeper in the tissue than a lens without correction collars. This is important for minimizing underestimation of spine counts; stubby spines only slightly protruded from dendritic shafts, and even
mushroom spines were sometimes too crowded to dissociate from each other. For spine counting, I selected dendrites extending parallel to the section to avoid trigonometric errors.

I estimated the total number of spines in the basal dendritic tree of an “average” cell at three representative retinotopic locations (0°, 1°, 20°) by calculating the product of the average number of dendrites and the average spine density for corresponding segments along the dendrites (Elston, 2001).

I estimated shrinkage of the brain tissue caused by perfusion in another adult monkey. Before perfusion, I implanted three pins into the brain at 4.00 mm intervals. After perfusion with the phosphate buffer and paraformaldehyde using the same protocol as in the main experiments, I measured the distances between pins. The distances (between pin 1 and pin 2, and between pin 2 and pin 3) were both 3.89 mm (2.5% shrinkage). Given the small degree of shrinkage, I made no shrinkage correction for the measured values.

3.3. Results

I visualized 202 layer-III pyramidal neurons in varying locations within the V1 from four hemispheres of three monkeys to examine whether their basal dendritic morphology differed depending on their location in the retinotopic map (Figure 3.4). I reconstructed the basal dendritic trees of these neurons to the full extent of their projections (Table 3.1). I then analyzed the density and number of dendritic spines by counting 18,496 individual dendritic spines. As I demonstrate below, the dendritic morphology of layer-III pyramidal neurons was uniform across 0–20° representation in the retinotopic map of V1.

3.3.1. Location of dye-injected neurons in the retinotopic map of V1

In V1 of the macaque monkey, the foveal region is represented in the anterior ventral part near the tip of lunate sulcus, a parafoveal field of ~8° is represented over the occipital operculum, and a visual eccentricity of 20° is represented within the calcarine sulcus (Figure 3.2E; Daniel & Whitteridge, 1961;Gattass et al., 1981; Van Essen et al., 1984). I verified this organization by mapping receptive fields in one case (CI14) before dye injection experiments. I recorded extracellular activity from individual V1 neurons in three widely spaced penetrations (P1-P3) over the occipital operculum (Figure 3.2A, B). P1 was near the anterior end of the operculum close to the tip of the lunate sulcus. P2 was in the middle of the operculum just below the extracalcarine sulcus, and P3 was near the mid-sagittal edge of the operculum. I determined the receptive fields (RFs) of three neurons in each of the three penetrations (Figure 3.2C). The horizontal eccentricities of the RFs in these penetrations were 0° (P1), 0.5° (P2), and 2.5° (P3). The mean RF size for each penetration was 0.115 deg² (P1), 0.077 deg² (P2), and 0.357 deg² (P3) (Figure 3.2C). The visual field representation and the RF-eccentricity relationship were consistent with those reported previously.

Our dye injections were aimed to three regions; one near the tip of the lunate sulcus that represents 0–0.5° (0° group), one in the middle of the occipital operculum that represents 0.5–2.5° (2° group), and one in the calcarine sulcus that represents >20° (20° group) (Figure 3.4). In the following analyses, I compared the dendritic and somal morphology between these regions.
Figure 3.4: Location of cells labeled by intracellular dye injection. Dye-injected cells are plotted on the lateral surface of the cortex (left) and on slice surfaces (right). Vertical gray lines indicate boundaries for estimated eccentricities of $0^\circ$, $0.5^\circ$, $2.5^\circ$, $8^\circ$, and $20^\circ$.

### 3.3.2. Morphology of basal dendrites and cell bodies in the primary visual cortex

Viewed from above, labeled neurons in tangential sections exhibited radially projecting basal dendrites (Figure 3.5A). I injected neurons with enough spacing so that the labeled dendrites of one neuron did not overlap with those from neighboring neurons. I were also careful to inject neurons whose cell bodies were located a few tens of microns below the surface of the section. I took this precaution to ensure that most of the labeled dendrites were entirely contained within the section. All labeled neurons were unambiguously identified as pyramidal neurons from their characteristic basal dendrites, a stump of a thick apical dendrites projecting towards the surface of the slice (i.e., toward the cortical surface) (Figure 3.5B), and numerous spines along the dendrites (Figure 3.5C).
Figure 3.5: Photomicrographs of 3,3′-diaminobenzidine tetrahydrochloride (DAB) product of Lucifer yellow-injected pyramidal neurons. (A) A low-power photomicrograph of dye-injected pyramidal neurons. Neurons were selected for injections with adequate spacing so that labeled dendrites did not overlap between adjacent labeled neurons. Most of the basal dendrites of the pyramidal neurons are included in the 250-µm thick tangential section. (B) A pyramidal neuron viewed at high magnification. The white arrowhead indicates the stump of the apical dendrite truncated at the slice surface. (C) Dendritic spines are clearly visible on dendrites.

Figure 3.6: Reconstructions of representative layer-III pyramidal neurons with receptive fields at 0°, 1°, and 20° of eccentricity. Cells were viewed in the plane tangential to the cortical surface. The neuron with the smallest basal dendritic field area at each location is illustrated on the left of each row and the neuron with the largest is illustrated on the right. The other neurons in each row represent 20% increments in dendritic field area (i.e., the 20th, 40th, 60th, and 80th percentiles).
Figure 3.7: Distributions of morphological parameters for layer-III pyramidal neurons. (A) Dendritic field area measured as an area of a convex hull over dendrites. (B) Number of principal dendrites. (C) Number of branches represented by the number of bifurcations of dendritic arbors. (D) Total length of dendrites. (E) Cell-body area. All parameters were measured from two-dimensional projections of reconstructed cells.

Figure 3.6 shows examples of the reconstructed dendritic morphology of the labeled neurons. For $0^\circ$, $2^\circ$, and $20^\circ$ groups, neurons are lined up separately with the smallest basal dendritic field area on the left (Min) and the largest on the right (Max). In between are neurons whose dendritic field areas fall in the 20th, 40th, 60th, and 80th percentiles. The extent, number, and branching complexity appear strikingly similar across the different eccentricity groups.

For quantitative comparison, I measured basal dendritic field area, number of principal dendrites, number of branches, total dendritic length, and cell-body area. I analyzed these morphometric parameters for the following reasons. The dendritic field size critically influences the geometrical range of input sampling (Malach, 1994). The total dendritic length determines the availability for synaptic contacts (Gray, 1959). The number of principal dendrites and number of branchings determine the number of components for dendritic computation (Spruston, 2008).
The basal dendritic fields of neurons in V1 were similar across visual eccentricities ($p = 0.012$, Kruskal-Wallis test; mean ± SD: $3.11 \times 10^4 \pm 0.65 \times 10^4 \text{µm}^2$ [$n = 122$], $2.76 \times 10^4 \pm 0.64 \times 10^4 \text{µm}^2$ [$n = 36$], and $2.92 \times 10^4 \pm 0.77 \times 10^4 \text{µm}^2$ [$n = 44$] for the $0^\circ$, $1^\circ$, and $20^\circ$ groups, respectively; **Figure 3.7A**). The number of principal dendrites was also similar across groups ($p = 0.21$; $3.7 \pm 0.65$, $3.78 \pm 0.64$, and $3.89 \pm 0.58$; **Figure 3.7B**). Similarly, neither the number of dendritic branchings ($p = 0.71$; $19 \pm 4$, $19.3 \pm 6.2$, and $19.8 \pm 5.3$; **Figure 3.7C**) nor the total length of dendrites ($p = 0.23$; $1.81 \times 10^3 \pm 0.41 \times 10^3 \text{µm}$, $1.76 \times 10^3 \pm 0.54 \times 10^3 \text{µm}$, and $1.93 \times 10^3 \pm 0.5 \times 10^3 \text{µm}$; **Figure 3.7D**) differed across groups.

I applied Sholl analysis to 2D reconstructions of the labeled neurons to analyze their dendritic branching geometry (Sholl, 1953). The Sholl profile of the neuron indicates the spatial distribution of dendritic arbors. The distance at which the number of intersections takes the maximum value indicates the location of the greatest number of dendritic branching.

The number of intersections between dendrites and Sholl rings gradually increased with distance from the cell body, peaked around 50 µm, and then declined slowly towards zero around 120 to 150 µm. I consistently found similar profiles between the regions and between the animals (**Figure 3.8**). The peak number of intersections showed a slight difference between the three regions ($p = 0.023$, Kruskal–Wallis test; $20.7 \pm 3.8$ for the $0^\circ$ group, $22.2 \pm 6.3$ for the $1^\circ$ group, and $22.8 \pm 4.7$ for the $20^\circ$ group). The peak value for the $0^\circ$ group was smaller than that for the $1^\circ$ group ($p = 0.01$, post-hoc Mann-Whitney U-test). The distance from the cell body at which the peak value occurred did not differ across groups ($p = 0.62$, Kruskal–Wallis test; $43 \pm 8.5$ µm, $42.5 \pm 7.6$ µm, and $43.8 \pm 7.6$ µm for the $0^\circ$, $1^\circ$, and $20^\circ$ groups, respectively).

![Figure 3.8: Profiles of dendritic branching visualized by Sholl analysis.](image)

Cell-body area differed between the three groups ($p = 0.002$; Kruskal-Wallis test; $130 \pm 23$ µm$^2$, $115 \pm 22$ µm$^2$, and $125 \pm 29$ µm$^2$ for the $0^\circ$, $1^\circ$, and $20^\circ$ groups, respectively; **Figure 3.7E**). Post-hoc analysis showed that cell-body area only differed between the $0^\circ$ and $1^\circ$ groups ($p = 3 \times 10^{-4}$, post-hoc Mann-Whitney U-test), while no significant difference was detected for the other comparisons ($p > 0.09$).
3.3.3. Dense sampling of neurons in the foveal representation

So far, our analysis has compared the dendritic morphology between three regions representing widely different visual locations (0°, 1°, and 20°). Because changes in visual field magnification are steepest near the foveal region, I might have missed subtle changes, if any, in the dendritic morphology of neurons in the foveal region. In the next experiments, I sampled neurons densely across the visual eccentricity near the fovea to determine if systematic changes occur in the dendritic morphology in this region.

In one experiment, I labeled 70 pyramidal neurons along an 8-mm line from the right hemisphere (Figure 3.9A). In this animal, I experimentally determined the retinotopic map by prior electrophysiological recording. The labeled neurons were from an estimated eccentricity of 0 – 0.5°. I plotted dendritic field area, number of branching points, and total dendritic length, against the distance from a reference cell located at 0° (open circle, Figure 3.9A). I found no correlations between this distance and the three morphological features; dendritic field area (Pearson’s linear correlation coefficient $r = 0.108$, $p = 0.14$; Figure 3.9B), number of branching points ($r = 0.151$, $p = 0.21$; Figure 3.9C), and total dendritic length ($r = 0.229$, $p = 0.030$, Figure 3.9D). In the other experiment, I visualized 40 cells in the left hemisphere of CI14 (Figure 3.9E-H) along a 20-mm line. In this hemisphere, I did not measure the retinotopic map with electrophysiology. Again, there were no correlations between the cortical location and dendritic structure ($r = -0.103, 0.153$, and 0.167, $p = 0.531, 0.354$, and 0.302 for dendritic field area, number of branching points, and total dendritic length, respectively).

Thus, even dense sampling of neurons near the foveal region did not reveal any systematic changes in the basal dendrite morphology of layer-III pyramidal neurons.

3.3.4. Density and total number of spines on basal dendrites

I counted dendritic spines to determine whether their density differed between the three regions of V1 (representing 0°, 1°, and 20°). I counted the numbers of spines in 10-µm segments along the basal dendrites and plotted them against the distance from the cell body (Figure 3.10). These spine distribution profiles were consistent across the three regions. The initial segment closest to the cell body was devoid of spines. The number of spines steeply increased at the next few segments, and reached a peak around 50 µm from the cell body. Dendritic spine density measured for the entire dendritic length did not significantly differ between the three regions ($p = 0.34$, Kruskal–Wallis test; 6.86 ± 3.42 spines/10-µm, 6.93 ± 3.78 spines/10-µm, and 6.74 ± 3.89 spines/10-µm for the 0°, 1°, and 20° groups, respectively).

I calculated the total number of spines in an “average” cell by calculating the product of dendritic length and spine density (the dot product of the Sholl profile and the spine density profile; Elston, 2001). On average, basal dendrites of layer-III pyramidal neurons had 1,119 ± 158 spines at 0°, 1,109 ± 205 at 1°, and 1,196 ± 199 at 20°. The estimated values for each area/monkey are plotted in Figure 3.11. These values were similar between groups ($p = 1.00$ for 0° vs. 1° groups, $p = 1.00$ for 1° vs. 20° groups, and $p = 0.64$ for 0° vs. 20° groups; random permutation test; Bonferroni-corrected).
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Figure 3.9: Analysis of dendritic structure from the fovea to the parafovea. Panels A-D and E-H indicate results from the right and left hemispheres of C114, respectively. I injected dye into a large number of pyramidal neurons distributed over a region covering visual fields from the fovea to the periphery to see any specialization in this region. (A, E) Each filled circle indicates the location of labeled cells. The open circles are the point-of-reference cells. P1 and P2 indicate the penetration points in the visuotopic mapping experiment (see Figure 2). (B-D, F-H) The dendritic field area, number of branching points, and total dendritic length are plotted against the distance from the reference cell.

Figure 10: Density profiles of spines along a dendrite. The dendrite was divided into 10-µm segments, and spine density was calculated for each segment and tallied along the dendrite. Dendritic spine density peaks at 40–50 µm from the cell body and decreases toward the distal tip.
3.3.5. Dendritic morphology of layer-III pyramidal neurons in area V4

I extended our analysis to cortical area V4 to investigate whether dendritic morphology in this intermediate stage of the ventral visual pathway (Roe et al., 2012) is similarly consistent across the retinotopic map. I analyzed 32 pyramidal neurons in the dorsal part of V4 located on the prelunate gyrus (Figure 3.12A inset). The samples used in this analysis were from a previous study (monkey CI10, Elston et al., 2010). I injected dye into neurons along a 6-mm long line on the prelunate gyrus (Figure 3.12A) so that the labeled neurons encompassed a wide area of visual field representation. The eccentricities of RFs of these neurons were assumed to cover 5° to 15°field representation based on the previously reported retinotopic map in V4 (Gattass et al., 1988; Kolster et al., 2014) and the experiences in our physiological studies (Watanabe et al., 2002; Tanabe et al., 2005; Kotake et al., 2009). Neither dendritic field area, number of branching points, or total dendritic length correlated with the location of the neurons (Figure 3.12B-D, \( r = 0.256, p = 0.158 \), for dendritic field area; \( r = 0.388, p = 0.028 \), for number of branching points; and \( r = 0.323, p = 0.071 \), for total dendritic length). This suggests that as in V1, the extent and complexity of basal dendrites of layer III neurons do not depend on the RF eccentricity in V4.

![Figure 3.11: Estimated total number of spines on an “average” cell.](image)

The total number of spines in the basal dendritic trees of “average” pyramidal neurons was estimated from the product of dendritic branching (Sholl profile) and spine-density profiles. The Sholl profile and spine-density profile used to calculate the total number of spines are the averages over sampled neurons of corresponding subgroups and individual animals. The estimated values were similar across groups.

![Figure 3.12: Morphology of basal dendrites of area V4 neurons.](image)

I injected dye into layer-III pyramidal neurons of V4 in the prelunate gyrus. (A) Each filled circle indicates the location of labeled cells. The open circle is the point-of-reference cell. (B-D) The dendritic field area, number of branching points, and total dendritic length are plotted against the distance from the reference cell. Data are from the case CI10 in Elston et al. (2010).
3.4. Discussion

I compared basal dendrite morphology of layer-III pyramidal neurons between foveal (0°), parafoveal (1°), and peripheral regions (20°) in the retinotopic map of macaque V1. Morphological characteristics of basal dendrites and their spines, such as dendritic field area, branching and number of dendrites, dendritic length, spine density, and total number of spines per neuron were homogeneous across the regions I examined. The area of input sampling and the amount of inputs by a single layer-III neuron via basal dendrites were thus uniform across the 0°-20° portion of the retinotopic map. I suggest that the expanded representation of the central visual field in the retinotopic map is accomplished before layer IV neurons project to layer III, and not by specialization of dendritic morphology in layer-III pyramidal neurons.

3.4.1. Morphological homogeneity of dendritic morphology across eccentricities

The morphological differences across eccentricities within V1 were much smaller than those between V1 and other visual areas. For example, layer-III pyramidal neurons in V2, V4, and cytoarchitectonic area TEO, have average dendritic field areas 1.2, 1.8, 3.6 times larger than those in V1 (Elston et al., 2010). In contrast, the maximum difference ratio within V1 between the groups representing 0°, 1°, and 20° was less than 1.13 (between the 0° and 1° groups). The extensive sampling along lines measuring 20 mm over the surface of V1 did not reveal any systematic changes in morphology of basal dendrites (Figure 3.9). Layer-III pyramidal neurons in V1 maintain a constant field area of basal dendrites across the cortical surface representing visual field eccentricities from 0° to 20°. This is in sharp contrast to neurons in the retina, which systematically and drastically increase their dendritic field with increased eccentricity (e.g., Wässle et al., 1989 for horizontal cells in macaque; Watanabe & Rodieck, 1989 for ganglion cells in macaque and baboon; Dacey, 1993 for ganglion cells in human).

I did not perform cytochrome oxidase (CO) histochemistry on our samples to determine whether each of the dye-injected neurons was from CO-rich blobs or CO-poor interblobs (Wong-Riley, 1979). Elston and Rosa (1998) previously reported that layer-III pyramidal neurons have a larger basal dendritic field area in CO-blobs (27.0 × 10^3 µm² on average) than in inter-blob regions (20.1 × 10^3 µm² on average). This raises a concern that if I sampled neurons with a systematic bias toward blobs or inter-blobs, differences in dendritic morphology across the eccentricities would be canceled out. However, the size and density of CO-blobs is constant within this portion of the retinotopic map of V1 (Farias et al., 1997; Adams & Horton, 2003). Furthermore, the blob size is in the same order as the size of V1 neurons; the diameter of blobs is about 269–281 µm (Farias et al., 1997), and the dendritic field diameter of layer-III V1 neurons is about 200 µm (Elston & Rosa, 1998; Elston et al., 2009; present results). It is therefore unlikely, if not entirely ruled out, that biased sampling occurred in our experiments and affected our conclusion.

The cortical depth of the sampled neurons could also have affected our results because layer II/III pyramidal neurons that are further from the cortical surface have longer basal dendrites (Larkman & Mason, 1990 for rodents). To make sure that the depth of injected cells was comparable among the cells, I selected neurons only immediately above layer IV for injection by confirming the granular appearance of layer IV under the microscope (see Materials and Methods). I also verified that the next section contained layer IV by staining for Nissl substance with Cresyl Violet. This two-step verification procedure mitigated the artifact due to variation in the cortical depth of injected cells.
3.4.2. Anatomy of expanded central representation in V1

The central visual representation gradually expands along the retino-geniculo-striate pathway (Perry & Cowey, 1985). Beginning in retina, RGCs are densely packed in the central visual field. RGC density peaks at the fovea and rapidly decreases toward the periphery (Perry & Cowey, 1985; Silveira et al., 1989; Adams & Horton, 2003). Representation of central vision is 3.5 times greater in the LGN than in the retina (Connelly & Van Essen, 1984; Adams & Horton, 2003), and another 6 times greater in V1 than in the LGN (Adams & Horton, 2003). In this way, the magnified central visual field in V1 results from a series of process along the retino-geniculo-striate pathway.

At least two potential anatomical mechanisms explain the divergent projections. One is that neurons at each earlier region in the pathway arborize their axons more extensively if they represent the central region than if they represent the peripheral regions (Figure 3.1A: afferent specialization hypothesis). Another is that neurons representing central vision receive broader connections from each earlier stage because of greater dendritic branching (Figure 3.1B: dendrite specialization hypothesis).

In the present study, I tested the dendrite specialization hypothesis along the projection from layer IV to layer III in V1. I demonstrated that across visual eccentricities of 0° to 20°, layer-III pyramidal neurons in V1 extend their basal dendrites to a similar extent (Figures 3.7–9) and receive a similar number of inputs within their basal dendritic branches (Figures 3.10, 3.11). Thus, dendrite specialization according to eccentricity does not occur in layer III of V1. It remains to be determined whether stellate cells in layer IV exhibit any systematic changes in dendritic morphology across the retinotopic map.

Regarding the axon specialization hypothesis (Figure 3.1A), there are two possible sites for specialization: LGN axons and layer IV stellate axons. Florence and Casagrande (1987) labeled geniculostriate axons by injecting horseradish peroxidase into nocturnal primate galagos, and found that the axonal arbors spread 2 times wider in the central visual region than in the peripheral visual region. Whether diurnal primates such as macaques also exhibit afferent specialization needs to be determined. Further, no study has yet compared the spatial extent of axon arborization of layer IV stellate cells between central and peripheral visual representations.

3.4.3. Cortical hierarchy vs. rostrocaudal position

Pyramidal neurons in layer III of higher cortical areas have a larger and more complex structure than those in lower areas. The higher in the processing hierarchy, the longer the dendrites and the more numerous the branches (Elston & Rosa, 1997, 1998; Amatrudo et al., 2012), and the larger and more extensive distribution of horizontal axon patches (Lund et al., 1993; Yoshioka et al., 1996; Fujita & Fujita, 1996; Tanigawa et al., 2005). Both dendrites and axons of pyramidal neurons change their morphological features postnatally with area-specific growth profiles. The profiles depend on the position of the area in the cortical hierarchy (for macaque monkeys: Elston et al., 2009, 2010; Wang et al., 2016; for marmoset monkeys: Oga et al., 2013; Sasaki et al., 2015). In these analyses, comparison between areas was made without paying attention to retinotopic representations.

Our analysis on V4 (Figure 3.12) suggests that as in V1, dendritic extent and complexity do not depend on the RF eccentricity in V4. The findings in V1 and V4 together indicate that previously documented differences in basal dendrite morphology between cortical areas did not likely result from unintentional sampling bias from a particular visual eccentricity. Rather, they provide strong support for the claim that the size and branching complexity of layer-III pyramidal neurons differ across different cortical areas (Elston et al., 1996; Elston & Rosa, 1997, 1998; For a review see Elston & Fujita, 2014).

Although the inter-area difference has often been interpreted to reflect the cortical hierarchy, Elston et al. (1996) raised another possibility that gradual changes in the dendritic arbor may reflect the
rostrocaudal position of the labeled neurons. They injected neurons along a rostro-caudal line in the posterior portion of visual cortices in the marmoset. The injected region included secondary visual cortex (V2), the dorsolateral area (DL), and the fundus of the superior temporal area (FST). They found that the gradual change in dendritic size was well fit by a single regression line. I, however, have shown here that neurons sampled from a region covering 20 mm rostrocaudally within V1 were uniform in size and branching of basal dendritic arbors, and in density and total number of spines (Figure 3.9), suggesting that the cortical hierarchy, rather than the rostro-caudal location, explains the previously reported inter-area differences in dendritic morphology.

3.4.4. Other morphological structures in V1

CO-blobs and ocular dominance columns (ODCs) are prominent anatomical structures observed across the cortical surface of V1. Early studies reported that the size of CO-blobs decreased and the density increased with increasing eccentricity in macaque monkeys (Horton, 1984; Livingstone & Hubel, 1984). However, later studies did not reproduce these findings and showed that the size and density of CO-blob were constant across V1 (macaque monkey, Farias et al. 1997; squirrel monkey, Adams & Horton, 2003). Unlike CO-blobs, ODCs vary their width with eccentricity. ODCs are wider in regions representing central visual field than in regions representing peripheral visual field (LeVay et al., 1985; Horton & Hocking, 1996a, b). On top of this retinotopy-dependent variation, the width of ODCs exhibit striking inter-individual differences (Horton & Hocking, 1996b). When compared between the corresponding retinotopic portions of V1 of different animals, ODCs could exhibit up to a two-fold difference in their width. Basal dendrites of layer III neurons did not exhibit such differences between different retinotopic locations or between individuals. These findings together suggest that layer III pyramidal neurons in the peripheral field may combine binocular inputs more readily than neurons in the central visual field. It would be interesting to compare the distribution of ocular dominance index between the two regions (Hubel & Wiesel, 1962).

Dendrites of spiny stellate neurons in layer IVCα and IVCβ remain in their home ODC where their cell body reside (Katz et al., 1989). Dendrites of pyramidal neurons in layer II/III cross over the CO-blob border (Malach, 1992; Hübener & Bolz, 1992). It is unclear whether dendrites of layer III pyramidal neurons care or ignore, i.e., remain inside or extend over, the border of ODCs. If they do care, peripheral neurons embedded in narrower ODCs would have smaller dendritic diameter than central neurons embedded in wider ODCs. As we showed in the present study, there was no detectable difference across the regions representing 0° to 20°. Layer III pyramidal neurons likely spread their branches across the border of ODCs. Functional specificity may be substantiated by finer organization of dendritic spines and axonal arborization.

3.5. Conclusion

I present evidence for morphological uniformity of dendrites of layer-III pyramidal neurons across visual eccentricities in V1. Our morphological analysis at the spine level also revealed geometric uniformity in the sampling of synaptic inputs by the basal dendrites of these neurons. The uniform dendritic convergence of information through layers IV to III in V1 suggests that the greater cortical representation of central vision is not ascribable to specialized morphology of pyramidal neurons in layer III, but is likely the result of a cumulative process that occurs earlier in the retino-geniculo-striate pathway.
3.6. References


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Chapter 4. Structural spine variation among cortical areas

In Chapter 4, I report that the pyramidal cells exhibit morphological variety at even spine level. Spine head of pyramidal cells in association cortices are varied in size. The evidence indicates that pyramidal cells conduct plasticity of the neural network by not only a greater number of spines but also plasticity of each spine.

Part of this chapter is under preparation for the publication.

4.1. Introduction

Primate central nervous system specializes its morphological and electrophysiological property. Layer III pyramidal cells in association cortices have a larger number of dendritic spines on extended and complex dendritic trees than primary sensory areas (Elston and Rosa, 1998; Rosa et al., 2000). Membrane property of pyramidal cells in prefrontal cortex suit temporal summation of synaptic inputs, whereas it of pyramidal cells in primary visual cortex allow them to follow high rate change of input (Amatrudo et al., 2012).

Dendritic spines are small protrusions, which exist on the dendrites of excitatory neurons in central nervous system (Cajal, 1888). Most spines have an excitatory synapse (Gray, 1959; Arellano et al., 2007). The spine morphological properties, such as head volume, neck length, and neck diameter, determine its physiological properties. Spines with larger heads have a wider postsynaptic density (Arellano et al., 2007), and produce larger mEPSP (Matsuzaki et al., 2001). Spine neck biochemically compartmentalizes spine head from the dendritic shaft. This filters synaptic input (Noguchi et al., 2005; Araya et al., 2006).

In this study, I quantified the spine head size in the primary visual area (V1), cytoarchitectonic area TE, and walker’s area 12 (Walker, 1940), which are the representatives for the primary sensory, sensory association, and executive cortices. The spine head was much more varied in size in area TE and area 12 than in V1, suggesting that neurons in association cortices are specialized to be more plastic than neurons in lower cortical areas. Here I performed a systematic study of the dendritic spine of the pyramidal cells in the primary sensory, sensory association, and prefrontal areas.

4.2. Materials and Methods

4.2.1. Animals and dye injection to individual pyramidal cells

Four male cynomolgus macaques (Macaca fascicularis) aged 4.5–7.5 years were used in the experiments. All were raised at Shiga Medical School (Otsu, Shiga, Japan; Table 1). The animal experiment committee of Osaka University (Suita, Osaka, Japan) approved the protocols for animal care and experimentation, which were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (DHEW Publication No. (NIH) 85–23, Revised 1996, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205, USA).

The intracellular dye injection techniques procedures have been described in detail in previous studies (Elston and Rosa, 1997). The animals were overdosed with sodium pentobarbital (Nembutal, >75 mg/kg, i.v.; Dainippon Sumitomo Pharma, Osaka, Japan). Animals were perfused with 0.1M phosphate buffer saline following 4% paraformaldehyde. Tissue blocks were then taken from the exposed portion of the occipital lobe (V1; corresponding approximately to the central 7° in the visual representation; (Daniel and Whitteridge, 1961), the inferotemporal cortex, and the prefrontal cortex (12vl; Preuss and Goldman-Rakic, 1991).

Sections for HVEM analysis were cut transversal to the cortical surface with the aid of a microslicer (Vibratome series 1500, Vibratome Company, St Louis, MO, USA). Tangential sections were prepared for the light microscopic analysis. I cut the blocks into 250-μm. The sections were first incubated in a solution containing 10–5 M of 4,6-diamidino-2-phenylindole (DAPI; D9542, Sigma-Aldrich Corp., St Louis, MO, USA) in PB for 3 min and mounted between Millipore filters (AABG02500, EMD Millipore Corp., Billerica, MA, USA) to make the section flat. The slice preparation was then mounted in a plastic dish on a fixed-stage microscope (Eclipse FN1; Nikon, Tokyo, Japan) and the preparation visualized with UV/blue excitation (380–420 nm).
Figure 4.1: Dendritic spines in V1 and area TE. The dendritic spine was much uniform in V1 (Left column) than in TE (Right column). Pyramidal cells in TE exhibited filopodia-like (head-less spine) (arrowheads), large mushroom type, and thin spines, while V1 cells lacked the variety of the spines. Scale bar = 10 μm.

DAPI-labeled neurons were injected with Lucifer Yellow (Lucifer Yellow CH dilithium salt, L-0259, Sigma-Aldrich Corp.; dissolved in 0.05M Tris buffer) under visual guidance with continuous current (up to 20 nA). I distinguished pyramidal cells from other neurons with following criteria. Neurons have thicker process than other processes. The thickest process extends to the cortical surface. These neural processes have dendritic spines.

Once injections were completed for a suitable number of neurons, the slice was processed for a light-stable reaction product by a photo-conversion technique (Lübke, 1993). The sections were incubated in a fresh solution containing 0.5 mg/mL 3,3-diaminobenzidine tetrahydrochloride (DAB, D5637, Sigma-Aldrich Corp.) in 0.1M PB for 30 min. After the preincubation, the DAB solution was replaced with an ice-cooled DAB solution. The tissue was then illuminated with excitation to Lucifer Yellow (430 nm) until dendritic segments and spines were visualized with DAB. During illumination, the DAB solution was rinsed off every 15 min and replaced with a fresh DAB solution. Tissue was rinsed in 0.1M PB.

4.2.2. Preparation for electron microscopic observation

DAPI-labeled tissue was incubated in a fixative (2% glutaraldehyde, 2% paraformaldehyde in 0.1M PB) for one overnight at 4 °C. The tissue was kept in 2% OsO₄ in 0.1M cacodylate buffer (Na(CH₃)2AsO₂ • 3H₂O; pH 7.4) for 2 hours at 4 °C after tissue was rinsed for 6 times in 0.1M PB. Tissue was then rinsed off with 0.1M cacodylate buffer for 6 times after the fixation.

Tissue was dehydrated with graded ethanol (50%, 70%, 80%, 90%, 99.5%, and 100%) 10 min each, and incubated in propylene oxide for 10 min for two times. Tissue was incubated in 50% Quetol 812 solution in propylene oxide for one overnight. The solution was replaced with 100% Quetol 812 and stored for 3–8 hours. Tissue was then stored in an oven at 60 °C for 72 hours.
The plastic embedded tissue was trimmed and cut at 4 µm thick with a diamond knife (histo 45, Diatome AG, Biel, Switzerland). The section was mounted on an ethylene dichloride coated grid. A solution containing gold colloid (60 nm; GC60, BBI Solutions, UK) was dropped on both sides of the grid. Then grid was coated with carbon for the purpose of reinforcement and avoiding the electric charge.

4.2.3. Observation in high voltage electron microscope

The advantage of light microscopic observation is a wider field of view and deeper focus than conventional electron microscopy (EM). The conventional EM, in contrast, has higher resolution than LM. It is difficult to quantify the fine morphology of the dendrite or dendritic spine shape in both LM and EM, because LM does not have enough resolution for quantifying submicron structure, and only thinner sections are observed with EM, so that the experimenter has to reconstruct the structure from numerous serial sections.

Dendritic segments in the 4-µm thick section were observed with a high voltage electron microscope (H-3000, Hitachi, Japan) in Research center for ultra-high voltage electron microscopy at Osaka University. The sections were observed in an HVEM operated at 2,000 kV. Projection images were recorded using a CCD camera (4,096 × 4,096 pixels; TVIPS, Germany) at 3,000 or 6,000× magnification. Tilted series pictures were prepared by tilting the specimen stage from -60 to +60 degrees at 1-degree interval. Images were denoised by the median filter and homogenized by high-pass filter by image process software (Photoshop, Adobe Systems Incorporated, CA, USA). Tomography was processed by IMOD (Kremer et al., 1996; Mastronarde, 1997). Dendrite and dendritic spine structure were reconstructed from the tomographic image with the aid of Neurolucida (MBF bioscience, Williston, VT, USA). Section shrinkage, which was due to electron beam emission, was corrected after I reconstruct dendritic structure by multiplying shrinkage correction factor calculated based on the thickness of topographical image.

Spines were classified to subtypes with following rules (Harris et al., 1992). Thin spine: the spine length (distance from the dendrite to the spine tip) is larger than 3 times of neck diameter. Mushroom spine: the spine head is larger than 10 times of neck diameter. Stubby spine: difference between the neck diameter and the spine length less than 10%. Branched spine: spine neck is branched. Filopodium: the protrusion has no constriction.

4.2.4. Light microscopic analysis

I prepared sections for LM analysis apart from sections for HVEM analysis. The analysis in HVEM only enables us to visualize ten microns so that it may produce biased data. With the aim of avoiding this bias from small and local sampling, I analyzed dendrites from proximal to distal part with LM. In this analysis, after I injected pyramidal neurons with Lucifer Yellow, immunohistochemistry has been processed to make sections light stable (see (Elston et al., 2009; 2010)).
Figure 4.2: Analysis of a dendritic segment with a high voltage electron microscope. A series of photographs were taken with changing stage angle from -60° to +60° (A). Tomographical images were computed from the photographs (B). The dendritic segment was reconstructed in 3D by tracing contours of tomographical slices (C). Spine head volume and neck length was quantified (D). Red arrowheads indicate the dendritic spines. Blue arrowheads indicate the dendritic shaft.

I focused our analysis on basal dendrites of layer III pyramidal cells. We quantified a diameter of over 1,500 spine heads by the following method. I chose horizontally extending dendrites and took Z-stack images with 0.13-µm step with 100× objective (N.A. = 1.49; CFI Apo TIRF 100×, Nikon, Tokyo, Japan). The z-stack image was deconvoluted with point-spread-function of the object lens with the aid of NIS-Elements Ar (Nikon, Tokyo, Japan). All light microscopic photographs were taken through a green interference filter (peak wavelength = 550 nm).

I measured a line luminance profile from Z-stack image to quantify the spine head diameter to exclude subjectivity. I fitted inverted Gauss function to the luminance profile and took standard deviation value as the diameter of spine head.

4.3. Results

I designed the experiment to quantify the spine geometry of the layer III pyramidal neurons of the macaque monkey and compare the parameters between cortical areas, including executive prefrontal cortex (PFC), visual association (TE), and the primary visual cortex (V1). I reconstructed 200 basal dendritic spines of individually labeled layer III pyramidal neurons of macaque monkeys by HVEM tomography and measured 1,982 spine head diameter by light microscopic analysis. These analyses demonstrated the broader distribution of dendritic spines in association cortices (TE and PFC) than in the primary visual cortex (V1).
I reconstructed 12 dendritic segments in 3D from V1, TE, and area 12 by HVEM tomography technique. I analyzed 24 spines from V1, 72 spines from TE, and 142 spines from area 12. I quantified spine head volume, surface area, neck length, and neck diameter from the reconstructed spines. Distribution of spine head volume was broader in association cortices (area 12 and TE) than in V1 (p < 0.05, F-test). Spine head volume in V1, TE, and area 12 were 0.0594 ± 0.0268, 0.0643 ± 0.0399, and 0.0534 ± 0.0417, respectively (Figure 3).

Figure 4.3: Distribution of spine head volume quantified with HVEM. Dendritic spine heads were much varied in association cortex (TE and PFC) than in V1. Asterisks indicate statistical significance (p < 0.05; F-test).

HVEM analysis has advantages in the resolution, but I can only observe 10-20 µm of the dendritic segment, whereas light microscopic analysis enables whole dendrite analysis. Light microscopic analysis has a disadvantage in the resolution, but I could observe a large number of samples. For that reasons, I measured 486 spine head diameter from V1, 1,045 spines from TE, 451 spines from PFC. Spine head widely distributed in TE and PFC than in V1 (p < 0.05; bootstrap test for S.D.; Fig. 6A). Mean values of spine head diameter were also larger in TE and PFC than in V1 (p < 0.05; Mann–Whitney U-test). Accumulated distribution function of TE and PFC rose up faster and saturated slower than the function of V1.

I investigated a tendency of spine head, whether spine head size increase or decrease as the distance from soma increase because previous studies reported that dendritic spine size in apical dendrites increases systematically as its distance from soma increase. To confirm that whether those systematic changes occur in the cerebral cortex, I divided the dendritic segment into 30 µm long, and compared mean spine head size of a segment among these of different segments. Dendritic spine head size was not significantly different regardless of a distance from the cell body (p > 0.05; Kruskal–Wallis test).

A resolution of light microscopy mainly depends on a numerical aperture of an objective lens, the wavelength of light, and diffusion of the specimen. To clarify our optics for LM analysis, I observed a dendritic segment with LM and HVEM (Fig. 7A for LM, Fig. 7B for HVEM). I plotted the relationship between length determined by LM and length by HVEM (Fig. 7D). In LM analysis, spine diameter that was larger than 0.45 µm was well estimated in our optics. Most spines (X% in V1 and Y% in TE) were well estimated in their head diameter.
Chapter 4. Structural spine variation among cortical areas

Figure 4.4: Light microscopic quantification of spine head diameter. The spine head diameter was quantified by calculating a half width of a line luminance profile which crossed spine head (A). An upside-down Gauss function was fit to the luminance profile, and then a standard deviation was adopted as the diameter of spine head (inset). Cumulative distribution functions were plotted. The function of V1 slowly raised and crossed to the function of area TE and PFC. Histograms of spine head diameter were inset. Spine head diameter was evaluated every 25-μm segment and plotted as a function of the distance from soma (B).

Figure 4.5: Accuracy evaluation of light microscopic analysis. Spine head size was measured with light microscope (LM) and HVEM to test the resolution of the data at this condition. Length longer 0.4 μm was measured well by both LM and HVEM, while length shorter 0.4 μm are overestimated in LM.

4.4. Discussion

I designed the current study to compare spine morphology of layer-III pyramidal neurons in the primary visual (V1), cytoarchitectonic area TE, and prefrontal cortex (PFC; 12vl) of the macaque monkey. I choose to examine these three areas because they are the representatives of primary sensory, sensory association, and executive cortex. Spine head was much more varied in size in TE and area 12 than in V1.
4.4.1. Technical considerations

In this study I applied light microscope and HVEM to quantify microstructure of the spine. Both of the analyses are complementary: it was possible in HVEM to reconstruct spine structures of small population at nano-meter scale, while in contrast I could obtain large number of samples in light microscopic analysis at sub-micron accuracy (Figure 4.5). The distribution wideness of spine head volume at association cortices by HVEM (Figure 4.3) well represented the impression of light microscopic observation (Figure 4.1). The light microscopic quantification supported the sample smallness. Although it was not shown in light microscopic analysis that existence of smaller spines in association cortices—the cumulative distribution functions of the spine head diameter were inseparable below 0.5 µm (Figure 4.4A), the optical limit resulted this inseparation (Figure 4.5).

4.4.2. Number of spines and spine variation increase network storage capacity

Pyramidal cells in the primary visual cortex have 700 basal dendritic spines on average, whereas cells in TE and PFC have over 5,000 spines (Elston and Rosa, 1997; Rosa et al., 2000; Elston et al., 2009). In addition to the number of synaptic contact site, I have shown that size of spine head is much more varied in association cortices (TE and PFC) than in V1, suggesting that variation of synaptic input is larger in association cortices than in V1. These two morphological specializations in association cortices enlarge diversity of synaptic connection. Thus both larger number and wider variation of synaptic input contribute the higher capacity of the neural circuits (Chklovskii et al., 2004).

4.5. Conclusion

The present data showed that cortical pyramidal neurons are morphologically specialized at even synapse level. Pyramidal cells have longer and branched dendrite and a larger number of dendritic spines with varied synaptic efficacy in association cortices than in primary sensory cortex. It is suggested that these specializations realize higher synaptic plasticity of the neural circuits in association cortices.
4.6. References


Chapter 5. General Conclusion

In the current study, I provided the evidence that the morphology of the pyramidal cell dendrites had regional and layer-specific developmental profile, characterizing structural variation of the cells in the adult brain. The previous study (Elston, Oga, Fujita, 2009) showed that the development of layer-III pyramidal cells differ in dendritic branches, spine density and the total number of spines between the primary visual (V1), temporal association (TE), and the prefrontal cortex (area 12). I expanded the study into infragranular layer, showing that pyramidal cells in V1, as like as layer-III, pruned the dendrites and spines than they grow them, while the growth exceeded the pruning in TE and area 12. The peak density and the total number of spines peaked 3.5 months of age in TE and area 12 in layer V as in layer III. In contrast, V1 had a peak at 3-week old, which was earlier than that of layer-III (3.5-month old) (Chapter 2). The observation by ultra-high voltage electron microscope revealed that, in the adult cortex, the microstructure of the dendritic spines also differ between areas (Chapter 4). Although pyramidal cell dendrites in the different area exhibit different feature, dendritic branches were homogeneous within V1, suggesting that not dendritic branches but the expansion of axon terminal had a dominant influence on the emphasis of cortical magnification factor in the central vision (Chapter 3).
Curriculum vitae

Tomofumi Oga, Ph. D. candidate
Graduate school of Frontier Biosciences
Email: oga@fbs.osaka-u.ac.jp
Osaka University
Yamadaoka 1-4,
Suita, Osaka, 565-0871, Japan

EDUCATION
2004-2009 Bachelor of Engineering, Osaka University
2009-2012 Master of Science, Osaka University, 2012 (advisor Ichiro Fujita)
“Morphology of dendritic spines of pyramidal cells in the primary visual cortex
and the inferior temporal cortex: analysis with ultra-high voltage electron
microscopic tomography”
2014-present Ph. D. Candidate in Neuroscience, Osaka University, expected graduation March 2017
(advisor Ichiro Fujita)

WORK EXPERIENCE
2012-2014 NTT-WEST (西日本電信電話株式会社)

PEER-REVIEWED JOURNAL ARTICLES FOR THIS THESIS
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SCHOLARSHIPS & ACADEMIC AWARDS


2015- Research Fellow of Japan Society for Promotion of Science