Exploring dendritic refinement of barrel cortex layer 4 neurons via high spatiotemporal-resolution *in vivo* imaging

Wang, Luwei

Doctor of Philosophy

Department of Genetics

School of Life Science

The Graduate University for Advanced Studies,

SOKENDAI

Introduction:

The specific arborization of dendrites determines the inputs the neuron receives, and the accurate dendritic pattern is formed through refinement during development in an activity-dependent manner. It is critical to understand how neurons refine their dendrite morphologies during development. To understand the dendritic refinement mechanism of cortical neurons at early postnatal stages, *in vivo* time-lapse imaging is useful. Recent studies in our laboratory that used two-photon *in vivo* time-lapse imaging (Mizuno et al., 2014; Nakazawa et al., 2018) have brought new perspectives to our understanding of dendritic refinement dynamics at the early postnatal stage. However, many details regarding the precise refinement features of cortical neuron dendrites are still largely unexplored because the imaging resolutions of these studies were not sufficiently high.

In my project, I improved the *in vivo* time-lapse imaging of the neonatal mouse brain to achieve higher spatiotemporal resolution. The spatial resolution for dendrite morphology imaging was improved by using a membrane-bound red fluorescent protein (RFP) (mRFP) instead of a regular RFP. The temporal resolution was improved from 8-hour interval to 1-hour interval in time-lapse imaging. In utero electroporation-based Supernova (Mizuno et al., 2014; Luo et al., 2016) was used to sparsely label barrel cortex layer 4 neurons with the mRFP, and TCA-GFP mice (Mizuno et al., 2014) were used to enable *in vivo* visualization of the barrel map. Then, I imaged dendrites of the same layer 4 neurons for 8 hours at postnatal day (P) 4, which is in the middle of the dendritic refinement process. Neurons labeled with the mRFP revealed more precise dendritic morphologies compared to the regular RFP. Using the improved imaging system with accurate reconstruction of the *in vivo* imaged neurons' basal dendritic patterns, I showed the general refinement dynamics of the barrel cortex layer 4 neurons at P4. I detected

many short dendrite trees and short branches (tip segments) that emerged and disappeared within the 8-hour imaging session. I sometimes found that a tip segment emerged at a location similar to where another tip segment previously disappeared.

I concentrated on analyzing the refinement dynamics of dendritic tip segments as well as short dendritic trees. I classified excitatory layer 4 neurons into two groups according to the differences in their basal dendrite orientation toward the barrel center. By conducting quantitative analyses, I detected differences in refinement dynamics of basal dendrites between these two neuron groups. In addition, I carefully observed sites at which dendrites were retracted during the last hour of *in vivo* imaging sessions by *post hoc* high-magnification confocal images and found no evidence for pruning/degradation events in the dendritic retraction process.

My study has shown the general refinement dynamics together with some specific morphological features of the layer 4 neurons in the barrel cortex via a high spatiotemporal-resolution *in vivo* imaging system.

Materials and methods:

Animals

All experiments were performed according to the guidelines for animal experimentation of the National Institute of Genetics (NIG) and were approved by the animal experimentation committee of the National Institute of Genetics. TCA-GFP Tg mice (Mizuno et al., 2014) backcrossed from C57BL/6J to ICR more than four times and were intercrossed to obtain TCA-GFP Tg homozygous male mice. To obtain pups, ICR female mice were mated with the TCA-GFP Tg homozygous male mice. The day at which the vaginal plug was detected was designated as embryonic day E0.5 and E19.5 was defined as postnatal day (P) 0.

Plasmids

Flpe/FRT-based Supernova vectors: pK036.TRE-Flpe-WPRE (Luo et al., 2016); pK037.CAG-FRT-STOP-FRT-RFP-ires-tTA-WPRE (Luo et al., 2016), pK300.CAG-FRT-STOP-FRT-GAP43-tagRFP-ires-tTA-WPRE, and pK302.CAG-tagBFP-WPRE, were used in this study.

In utero electroporation

In utero electroporation was performed on TCA-GFP Tg heterozygous mice at E14.5 to label layer 4 cortical neurons. The pregnant mothers were anesthetized via intraperitoneal injection of triple anesthesia with the dose of 11 mg/kg body weight. The triple anesthesia contains medetomidine hydrochloride 0.75 mg/kg, midazolam 4 mg/kg, and butorphanol tartrate 5 mg/kg. DNA solution mixed with methylene blue was injected into the right lateral ventricle of embryos via a pulled glass capillary (DRM), and square electric pulses (50 mV; 50 ms) were delivered

five times at the rate of one pulse per second by a CUY21EDIT electroporator (NepaGene). For the Supernova-RFP expression, a solution containing pK036 (20 ng/μl) and pK037 (1 μg/μl) was used. For the Supernova-mRFP expression, a solution containing pK036 (20 or 40 ng/μl), pK300 (1 μg/μl) and pK302 (200 ng/μl) was used. The electric pulses were 3 times/embryo, and the current was 70-100mA / electric pulse. After the in utero electroporation, pregnant mothers were injected with an antagonist with the dose of 11 mg/kg body weight (containing atipamezole hydrochloride 0.75 mg/kg) and kept on a 37℃ heater until they recovered from anesthesia.

***In vivo* imaging and two-photon microscopy**

For high temporal resolution imaging of layer 4 neurons in the barrel cortex at P4, TCA-GFP pups, in which layer 4 neurons were sparsely labeled by in utero electroporation-based Supernova-mRFP.

A cranial window was made on the right half of the heads of those P4 pups over the barrel cortex. The cranial window surgery was performed as described previously (Nakazawa et al., 2018). After 1 hour of recovery on a 37°C heater with littermates, the pups with cranial windows were used for *in vivo* imaging. Those pups were anesthetized with 0.9-1.5% isoflurane and fixed to the microscope stage using a titanium bar during the *in vivo* imaging. A heating pad was used to keep pups warm. Images were acquired using an LSM 7MP two-photon microscope (Zeiss) with a W Plan-Apochromat 20x/1.0 DIC objective lens (Zeiss) and an LSM BiG detector (Zeiss). HighQ-2 laser (Spectra-Physics) at 1,045 nm was used in all experiments. GFP and RFP were simultaneously excited and emitted fluorescence was filtered (500−550 nm for GFP and 575−620 nm for RFP).

Histology and confocal microscopy

After *in vivo* imaging at P4, brain samples were obtained and fixed with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The brain samples were kept at 4℃ overnight and protected from light. For tangential sectioning, right hemispheres were flattened and transferred to 30% sucrose in 0.1 M PB overnight at 4℃. Tangential sections (100-µm thick) of the flattened cortices were made using a freezing microtome (ROM-380; Yamato).

Three-dimensional fluorescent images were acquired using a confocal microscope (TCS SP5; Lecia). Images of barrel patterns and IUE-labeled neurons were taken with an x10 lens. Images of fine structures of dendrites were taken with an oil immersion x63 lens.

Image analysis and quantification

IMARIS filament tracer (Bitplane) and Image J/FIJI (NIH) software were used to analyze the dendritic structure of individual neurons and barrels. Barrels and Supernova labeled neurons were confirmed by using Image J/FIJI (NIH). Thalamocortical axon cluster boundaries were defined as barrel edges and determined from the P4 mice confocal images of tangential sections. The barrel edge was determined by the contrast of thalamocortical axon signal intensities between the barrel center and septa. The distance from the neuron to the barrel edge is defined as the distance from the center of the soma to the nearest barrel edge. Here, I classified a neuron as an edge-located neuron if the distance from center of the soma to the barrel edge is less than 20 µm. Other neurons located within barrels were classified as barrel-center neurons. Neurons located on the

edge of main barrels (row A~E, arc 1~5) were used for analysis. The 180° barrel inside/outside boundary used for OBI analysis was decided as described in the previous paper (Nakazawa et al., 2018). Acquired three-dimensional images from a two-photon microscope were analyzed using the IMARIS Filament Tracer software (Bitplane). To keep the accuracy and efficiency of the data and analysis, I only analyzed those neurons with clear morphologies both during *in vivo* imaging and following confocal imaging. Dendrite morphology is a multi-level structure. A single dendrite is also known as a dendritic branch or segment. Dendrites that share a common origin from the cell body are on the same dendritic tree. The segments which located on the most distal part of dendritic trees are tip segments. The analyses of length change in 1 hour and calculation of dendritic OBI were done with the considering individual dendritic segments independently (Nakazawa et al., 2018). In some cases, one dendritic tree has segments toward to both inside and outside of the barre and could be classified differently into either IN or OUT groups.

To analyze the correlation of dendritic tip morphologies with its dynamics, I selected the most distal 5 μm part of the segment as the tip (T), and skip the adjacent 5 μm, then measured the shaft (S) 5 μm. Regions of interest of T and S were then determined by the mRFP-labeled dendrites at the resolution of the single pixel level. Initially, I picked out tip segments that showed distinguishable tip morphologies from their surrounding environment. Among the tip segments, samples were analyzed to fit the requirements of no overlapping with other dendrites or imaging noise, and at the same time, the segment length was longer than 15 μm to include 3 parts. I drew the ROIs according to the dendrite morphology under the high magnification in Image J/FIJI (NIH) software. The mRFP signal intensity (gray value) of ROIs was measured. The dendritic tip thickness index.

Statistical analysis

Statistical analyses were performed using GraphPad Prism, Image J/FIJI (NIH) and Microsoft Excel software. The quantification of dendritic segment refinement dynamics and the differences between high and low OBI neurons were done with Microsoft Excel and GraphPad Prism. The significance of the differences was assessed by the Mann-Whitney test, Wilcoxon matched-pairs signed rank test or binomial test. Values are given as means ± SE. The asterisks in the figures indicate the following: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. When $p>0.05$, it indicates as ns.

Results:

In this study, I aimed to investigate detailed dynamics and capture the possible transient developmental events of dendritic refinement more precisely. To understand those cellular aspects, I improved the *in vivo* imaging system to achieve high time resolution. To this end, instead of the previously performed 8-hour interval imaging (Nakazawa et al., 2018), I performed the imaging with a 1-hour interval. Additionally, I improved the spatial resolution of *in vivo* imaging, which was achieved by using a mRFP (Liu et al., 1994; Moriyoshi et al., 1996) instead of a regular RFP that was used in previous studies (Nakazawa et al., 2018, Mizuno et al., 2014). I sparsely labeled barrel cortex layer 4 neurons with the mRFP using the in utero electroporation (IUE)-based Supernova method (Mizuno et al., 2014; Luo et al., 2016). The precise dendritic morphologies of neurons that were labeled with the mRFP were clearly visualized *in vivo*. I imaged dendrites of the same neurons for 8 hours at P4, which is in the middle of the dendritic refinement process. With

the 1-hour interval imaging, I was able to catch subtle changes in basal dendrites including those of short-lived dendritic trees and branches. My imaging system also detected some transient morphological features in basal dendrites, which correlated with their behavior (elongation or retraction). Basal dendrites with a thinner tip often retracted in the following hour. Furthermore, layer 4 neurons whose basal dendrites showed higher and lower orientation bias exhibited different refinement dynamics. Therefore, my study revealed the detailed dendritic refinement process *in vivo* together with some specific features of the barrel cortex layer 4 neurons.

Discussion:

I improved the *in vivo* imaging for the neonatal mouse brain to a higher spatiotemporal resolution. I reconstructed the dendritic patterns *in vivo* imaged L4 neurons three-dimensionally. By analyzing them, I characterized the basal dendritic refinement dynamics of layer 4 neurons in the neonatal barrel cortex. I found that the high orientation bias neurons showed fewer emerged and transient dendrites than the low orientation bias neurons. Besides, the high orientation bias neurons preferentially extend or retract their existing dendrites, yet the low orientation bias neurons are involved more in forming new branches. My study also provided the first *in vivo* evidence for the positive correlation between the specific dendritic tip morphologies with their following refinement dynamics. My study that improved the neonatal *in vivo* imaging system may contribute to the understanding of the detailed dendritic refinement dynamics of cortical neurons.