Single-cell transcriptome analysis of mouse somatosensory cortex during postnatal circuit refinement

Kandasamy, Ramasamy

Doctor of Philosophy

Department of Genetics

School of Life Science

The Graduate University for Advanced Studies,

SOKENDAI

Abridged version of the thesis

Introduction

Proper functioning of the mammalian cerebral cortex requires precise interconnection of neurons, which arises through neural activity-dependent refinement in the postnatal stages. The molecular mechanisms of activity-dependent refinement are not well understood.

The layer 4 excitatory neuron in the mouse somatosensory cortex is a widely used model to understand molecular mechanisms involved in activity-dependent circuit refinement. In mice, the whiskers, which are hair-like tactile sensory organs, form a topographic map in layer 4 of the somatosensory cortex, known as the barrel map. Layer 4 excitatory neurons form a barrel-like structure and receive inputs from thalamocortical axons (TCAs), which occupy the barrel hollow. The barrel structures arise during the first postnatal week through an activity-dependent refinement process. Gene expression profiling is a promising approach for identifying candidate genes for molecular mechanisms of layer 4 neuron circuit refinement.

Layer 4 excitatory neurons in the mouse somatosensory cortex comprise of two morphologically distinct subtypes, namely spiny stellate and star pyramid neurons. Spiny stellate neurons exhibit asymmetric basal dendritic orientation to confine their dendrites to one barrel, while star pyramid neurons do not necessarily confine their dendrites to one barrel. This basal dendritic orientation of spiny stellate neurons arises during the first postnatal week through an activity-dependent refinement process. Even within spiny stellate neurons, those located on the barrel edge spiny stellate neurons) show higher dendritic dynamics as compared to those located in the barrel center. Given that different cell types exhibit varied response to activity, gene expression profiling at the level of individual cell types would give better insight to molecules that regulate barrel formation and development of dendritic orientation. To do this, in this study, I used single-cell RNA sequencing (RNA-seq) to identify cell types in layer 4 neurons and examine transcriptomic variation across cell types and between early (postnatal day 4, P4) and late (P7) phases of circuit refinement.

Materials and methods

Animals

All animal experiments were performed as per the guidelines for animal experimentation of National Institute of Genetics. Thalamocortical axon-green fluorescent protein (TCA-GFP) homozygous transgenic mice that were previously generated in C57BL/6 background (Mizuno et al., 2014) and backcrossed to ICR more than nine times were used. For timed mating, a female wild type (WT) mouse of ICR strain was put together with a male TCA-GFP homozygous transgenic mouse in the evening between 5:00 PM and 7:00 PM and vaginal plug formation was checked the following day in the morning between 11:00 AM to 11:15 AM. If a plug was observed, then that day was defined as embryonic day 0.5 (E0.5). E19.5 was designated at P0.

In utero electroporation

The pregnant mouse was anesthetized by injecting 10 µl / g body weight of combination anesthetic of medetomidine, midazolam and butorphanol (M/M/B: 0.3/4/5). Skin and abdominal wall were cut along the midline to expose the uterus. About 0.2-0.5 µl of plasmid solution containing 10% v/v Trypan Blue was microinjected into each lateral ventricle of embryonic mouse brain. Five square electric pulses (40 mV, pulse duration: 50 ms and inter-pulse interval: 950 ms) were delivered using an electroporator (CUY21SC; NepaGene). After electroporation, the uterus was replaced, and the abdominal wall and skin were sutured. Following completion of surgery 10 µl / g body weight of antagonist atipamezole was administered by intra-peritoneal injection and then the mouse was immediately placed on 37ºC heater for recovery.

Plasmid

The plasmid pK292.CAG–nuclear localization signal (NLS)–TurboRFP–WPRE was constructed in our laboratory. This plasmid was used for in utero electroporation at a concentration of 1 µg/µl to label layer 4 neurons.

Preparation of single-cell suspension from barrel cortex

Single-cell suspensions were prepared from P4 and P7 pups. The pups were sacrificed, and the brain was quickly transferred to ice-cold ACSF. The major barrels region was obtained from TCA-GFP mice by using a biopsy punch. A 0.75 mm inner diameter punch was used to obtain the major barrels region from brains of P4 pups and a 1.0 mm inner diameter punch was used to obtain the major barrels region from brains of P7 pups. After obtaining the tissue by punch, deeper layers, roughly layers 5 and 6, were removed using a spring scissors. To prepare single-cell suspension, the punched tissue was first subjected to enzymatic dissociation. Following this, the tissue was triturated.

Isolation of RFP-positive neurons

RFP(+) cells were obtained from cell suspension using fluorescence activated cell sorting (FACS). Target cells were chosen based on two criteria. The first criterion for selection was the intensity of forward scattered (FSC-A) and intensity of back scattered (BSC-A) light. This gating selects for cells with larger size and eliminates debris and dead cells whose membrane integrity is compromised. The second criterion for selection was RFP fluorescence intensity.

Single-cell library preparation and sequencing

Single-cell RNA-seq libraries were prepared by our collaborators using Chromium Single Cell 3' Reagent Kits v2 (10x Genomics) according to the kit instructions. The input number of cells for P4 and P7 were aimed towards achieving a recovery of 3,000 cells for P4 sample and 1,000 cells for P7 sample. The libraries were sequenced using NovaSeq 6000 sequencing system (Illumina) by our collaborators.

Data processing

Sequencing reads were obtained in FASTQ format. CellRanger v3.0 was used to align the reads to mouse genome (mm10/GRCm38 and GRCm38.93.GTF) that was modified to include the sequence of NLS turbo RFP. The gene expression is quantified as unique molecular identifiers (UMI) counts. Two rounds of clustering were performed using Seurat. Output from CellRanger was imported into Seurat and a Seurat class object was created. Prior to the first round of clustering cells were filtered based on quality statistics. This data after quality control was used

for first round of clustering using Seurat. For the second round of clustering, the Seurat object obtained from the first round of clustering was used as input. Neuronal clusters were identified as clusters expressing the neuronal marker *Rbfox3* (*NeuN*).

Monocle3 was used to perform trajectory analysis. Seurat objects from P4 and P7 were combined into a single Seurat object. This combined Seurat object was filtered to select for cells belonging to clusters P4A, P4B and P7E. This combined and filtered Seurat object which contains only layer 4 neurons from both P4 and P7 stages was used as input to Monocle3.

Protein interaction data for mouse (Mus musculus, version 11.0) were obtained from STRING database (Szklarczyk et al., 2019). Two proteins were considered as interacting if their 'combined score' was greater than or equal to 0.6. Cytoscape along with EntOpt layout was used to visualize the interaction network between differentially expressed genes.

Results

I labeled layer 4 neurons by in utero electroporation of RFP at embryonic day 14.5 (E14.5). The embryos were let to develop, and single-cell samples were prepared at P4 and P7. I accurately punched and obtained the barrel region by using TCA-GFP mice, which express membrane-bound enhanced GFP in thalamocortical axons and thus aid in visualizing the barrel map. The punched tissue was dissociated, and RFP-labeled cells were obtained by fluorescence-assisted cell sorting and single-cell libraries were prepared. Following sequencing and alignment of reads, gene expression matrix was obtained. I performed clustering and identified neuronal clusters. There were 862 neurons out of 1,141 total cells at P4, and 263 neurons out of a total of 710 cells at P7. All the neurons from both P4 and P7 samples were identified as upper layer neurons (layer 4 or layer 2/3) based on *Cux1* expression.

A second step of clustering was performed to identify subtypes within neurons. Four clusters (P4A to P4D) were identified. Based on *Rorb* expression and data from previous transcriptomic analysis (Fertuzinhos et al., 2014), I identified P4A and P4B as layer 4 and P4C and P4D as layer 2/3 neurons. Four clusters (P7E-7H) were identified among P7 neurons. P7E was identified as layer 4 neurons and the other three were identified as layer 2/3 neurons. At P7, layer 4 neurons were clustered to only one cluster (P7E), suggesting that layer 4 neuron subtypes may not be sufficiently distinguishable at P7. Trajectory analysis suggests that P7E is more closely related to P4A than to P4B.

Gene expression analysis revealed that, genes involved in ubiquitin proteasome system, genes involved in ATP synthesis and genes involved in regulation of mRNA are differentially expressed between subtypes of layer 4 neurons at P4. Upregulation of genes related to ubiquitin proteasome system, which plays an important role in plasticity-related mechanisms, and other plasticity related genes such as *Lamp1* in P4A suggests that P4A could be more plastic than P4B and would exhibit activity-dependent developmental changes. On the other hand, analysis between clusters P4A vs P7E and P4B vs P7E has identified genes that exhibit temporal dynamics during layer 4 neuron circuit maturation. I found that several genes encoding ribosomal subunits, which are essential for plasticity mechanisms dependent on local translation, are upregulated at P4. Genes related to calcium signaling, axon transport and synaptic vesicle release showed higher expression at P7.

Discussion

Here, I studied transcriptomic variance and dynamics in mouse barrel cortex layer 4 neurons at early and late stages of postnatal circuit refinement (P4 and P7) using single-cell RNA-seq. For the first time this study identified two molecularly distinct subtypes (clusters P4A and P4B) within layer 4 excitatory neurons at P4. While I could not decisively determine the nature of these two clusters, one possibility is that they correspond to spiny stellate and star pyramid neurons, and another possibility is that P4A and P4B correspond to edge-located and center-located spiny stellate neurons, respectively.

To understand the relationship between the clusters P4A, P4B and P7E, I used Monocle3 to infer the trajectory. The inferred trajectory placed P4A between P7E and P4B. This could be interpreted in two ways. In the first interpretation, P7E belongs to the same subtype as P4A and P4B belongs to a subtype distinct from both P4A and P7E. In the second interpretation, all the clusters P4A, P4B and P7E belong to the same subtype but at different levels of maturation.

Differential expression analysis revealed genes that vary across cell types and across development. Between P4A and P4B, most of the differentially expressed genes were upregulated in P4A and these genes are key players in plasticity. This suggests that P4A could represent a subtype that is more plastic and more sensitive to neuronal inputs than P4B neurons. Upregulation of ribosomal subunits at P4 as compared to P7 suggests important role for protein translation during early phase of circuit refinement.

In conclusion, in this study, I identified differentially expressed genes across cell types (P4A vs P4B) and across developmental stages (P4A vs P7E), which could be important candidate genes for molecular mechanisms behind circuit refinement in the somatosensory cortex. Moreover, for the first time this study has identified molecularly defined subtypes within layer 4 excitatory neurons.