# 論文の内容の要旨

Layer-specific Heterogeneity of Astrocytes in the Mouse Neocortex
（大脳新皮質アストロサイトの層特異的多様性）

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## Introduction

Astrocytes are the most abundant glial cells in the mammalian central nervous system (CNS), having unique anatomical localization that links blood vessels and neurons through their highly ramified processes. This localization allows astrocytes to provide metabolic support to neurons. They also participate in the information processing by regulation of neuronal activity through their physical connections and the release of gliotransmitters.

The mammalian neocortex is organized into a six-layered structure with distinct neuronal subtypes in each layer (Figure 1a). These neuronal subtypes exhibit unique morphological features, express different sets of molecules, project their dendrites and axons to different target regions, and serve different functions. Contrary to the well-studied heterogeneity of neocortical neurons, the diversity and properties of neocortical astrocytes still remain largely unknown. Indeed, astrocytes in layers II to VI have been considered to be homogeneous and are called "protoplasmic" astrocytes (Figure 1b). However, it is also possible that these astrocytes are heterogeneous and display layer-specific differences like neocortical neurons (Figure 1c). We therefore compared the morphology and gene expression of the astrocytes populating distinct cortical layers.

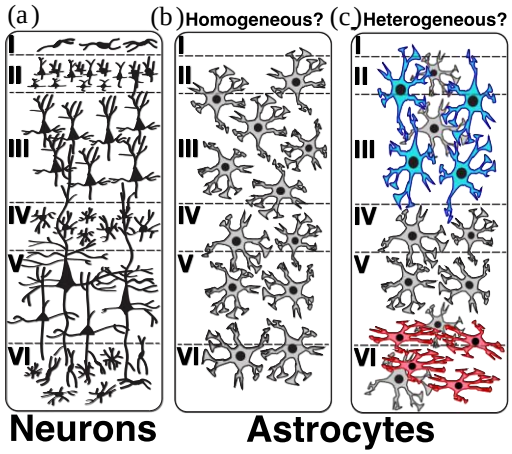
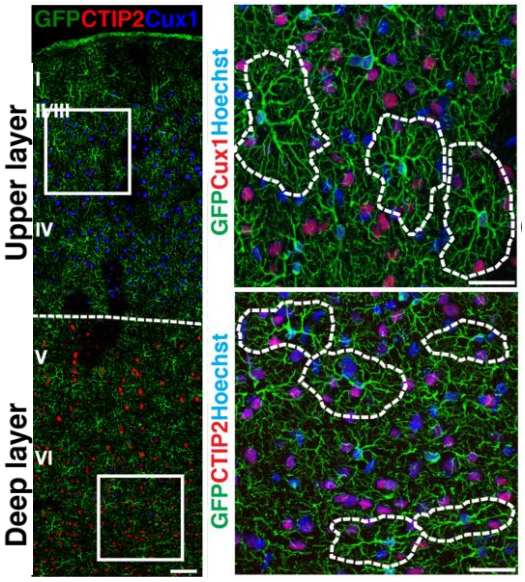



Figure 1: Do neocortical astrocytes display layer-specific heterogeneity?

## Results

### Layer-specific heterogeneity of neocortical astrocytes in morphology

First, we examined the morphology of astrocytes in distinct cortical layers by immunofluorescent staining of adult mouse brain sections to visualize either intermediate filaments, microtubules, or whole-cell structure of astrocytes. Intriguingly, we found morphological differences between astrocytes located in the upper and deep layers; most astrocytes in the upper layer (layers II/III and IV)




lie vertically to the brain surface, whereas most astrocytes in the deep layer (layers V and VI) lie horizontally (Figure 2). Also, the processes of upper-layer astrocytes are more ramified compared with those of deep-layer astrocytes. morphology demonstrated that astrocytes in different cortical layers are statistically different in terms of territorial volume and cell orientation (Figure 3). These results suggest the existence of layer-specific astrocyte subtypes.

Figure 2: Morphological differences between upper- and deep-layer astrocytes visualized by transgenic mice expressing Glast-EMTB-GFP, a GFP-tagged microtubule binding protein, driven by a glial specific (Glast) promoter. Scale bar = 25 μm.

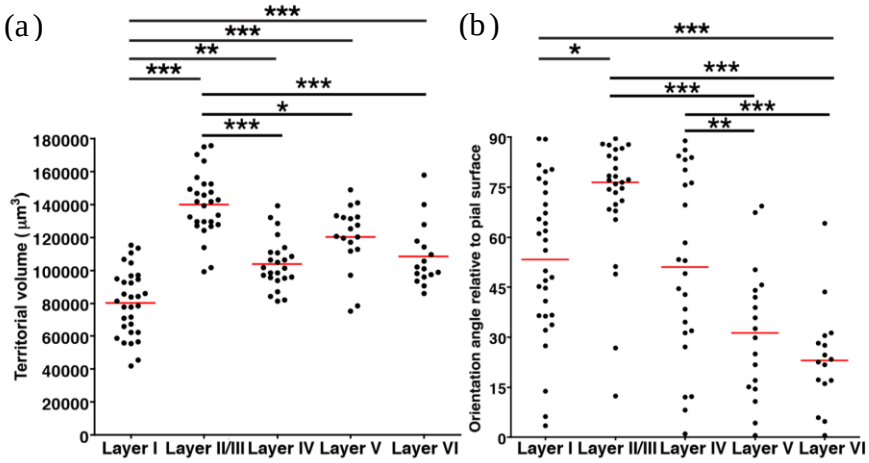
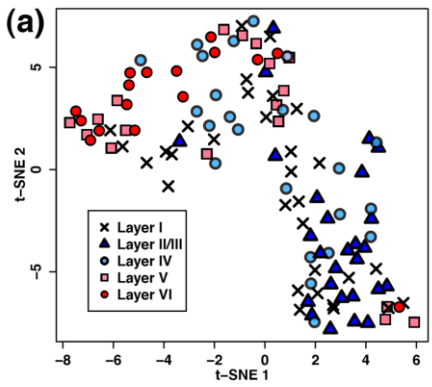



Figure 3: Quantification of territorial volume (a) and cell orientation (b) of neocortical astrocytes sparsely labeled in Glast-creER$^{T2}$:Rosa-CAG-LSL-tdTomato: Glast-EMTB-GFP mice (P65). One-way analysis of variance (ANOVA); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Given that astrocytes in different layers appear to exhibit their own distinctive morphologies, we further investigated a correlation between the morphological properties and the laminar position. Two independent cluster analyses, t-SNE (Figure 4a) and hierarchical analysis (Figure 4b), revealed that neocortical astrocytes are classified into groups based on 3D morphological features such as cell shape and cell orientation. Our cluster analysis showed a distinct morphological population, cluster D, resided primarily in layer I, consistent with previous studies reporting that layer I astrocytes are different from layers II/III-VI astrocytes in terms of molecular and electrophysiological properties. Interestingly, this analysis also showed laminar differences in layers II/III-VI astrocytes, with most of cluster A and cluster C astrocytes located in the deep and upper layers, respectively (Figure 4c). This layer-specific



distribution reinforces the notion that neocortical astrocytes exhibit layer-specific heterogeneity at least in terms of morphology.

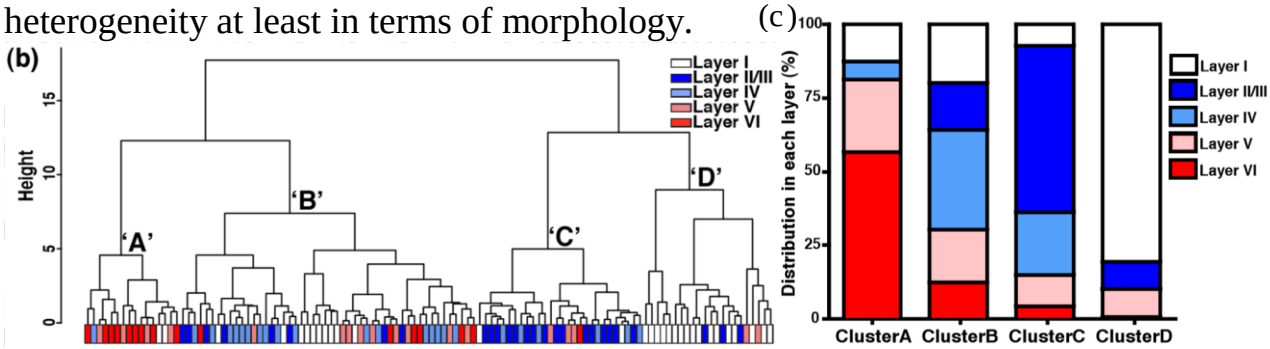



Figure 4: The classification analyses based on the 3D morphological features. (a) t-SNE shows the separated distribution of layer II/III astrocytes and layer V-VI astrocytes. (b) Hierarchical clustering reveals that neocortical astrocytes are classified into 4 clusters. Note that cluster A, cluster C, and cluster D astrocytes are enriched in layers V-VI, layers II/III-IV, and layer I, respectively (c).

## Layer-specific heterogeneity of neocortical astrocytes in gene expression

Next, to investigate whether neocortical astrocytes also exhibit layer-specific molecular heterogeneity, we compared the gene expression patterns of neocortical astrocytes. According to the morphological cluster analyses, astrocytes with different features locate asymmetrically in the upper and deep layers, thus we compared the gene expression profile of upper-layer astrocytes (ULAs) and deep-layer astrocytes (DLAs). ULAs and DLAs were collected by layer-dividing manual dissection and astrocyte isolation using FACS. Preliminarily, RNA-seq analysis revealed 1935 genes enriched in ULAs and 789 genes enriched in DLAs (n=1). Of these, we validated the expression differences of some interesting genes by quantitative PCR using astrocytes at postnatal day (P) 16-25 (Figure 5a) and at P11-12 (Figure 5b). ULAs and DLAs differentially express several genes that encode extracellular secreted proteins (Dkk3, an antagonist of Wnt signals; Chrdl1, an antagonist of BMP4), transporters (GlyT1, a glycine transporter; GluT2, a glucose transporter), enzymes (Adcy8, an adenylyl cyclase), and receptors that are known to be critical in the morphogenesis and axon guidance (Slitrk5, a member of SLIT and NTRK like family; EphA3, an Eph receptor). Consistently, in situ hybridization and immunostaining data also confirmed the enriched expression of Epha3 in ULAs and that of Adcy8 in DLAs. These results suggest that neocortical astrocytes also exhibit layer-specific heterogeneity in terms of gene expression.

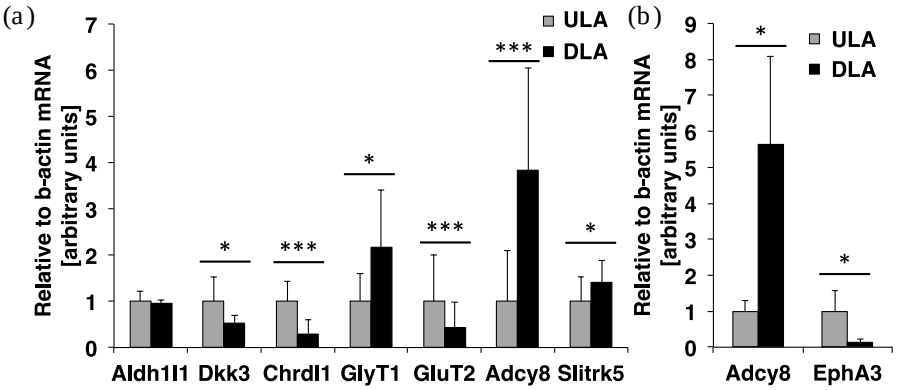
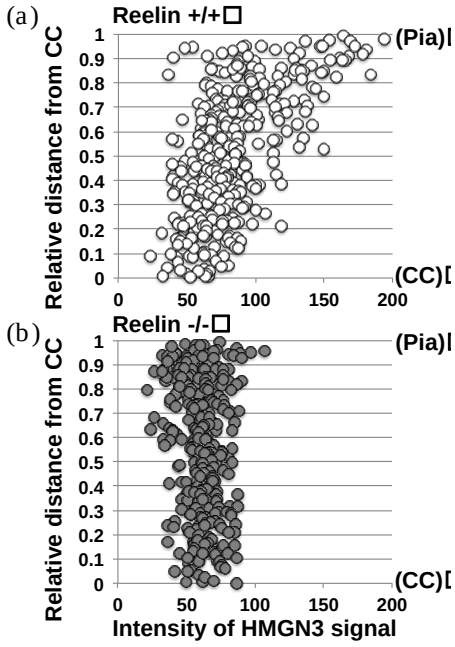



Figure 5: Differential gene expressions in upper-layer astrocytes (ULA, grey bars) and deep-layer astrocytes (DLA, black bars). (a) qPCR results at P16-25. (b) qPCR results at P11-12. Two-tailed paired Student's t-test; *, $P < 0.05$; ***, $P < 0.001$.

In addition to the differential mRNA levels, we also found the differential expression in protein levels of HMGN2 and HMGN3, which are known as critical transcription factors for astrocyte differentiation[1]. Quantification of immunostaining demonstrated that astrocyte HMGN3 protein expression shows graded expression, with highest signals in layer I and the upper part of layer II/III, and gradually decreasing along layers II/III-V, and nearly negative in layer VI (Figure 6a). These results substantiate the existence of layer-specific molecular heterogeneity of neocortical astrocytes.

## Disrupted layer-specific heterogeneity of neocortical astrocytes in reeler mice




We next wondered whether this layer-specific heterogeneity of astrocytes is still observed in reeler mutant mice, which exhibit laminar disorganization in cerebral cortex due to loss of the guidance protein called Reelin. Interestingly, the layer-specific expression of HMGN3 was not observed in reeler mice (Figure 6b). This finding proposes the possibility that layer-specific heterogeneity of neocortical astrocytes is influenced by exogenous factors such as the laminar structure of neighboring neurons and/or the secreted protein Reelin.

Figure 6: The layer-specific expression of HMGN3 protein was not observed in reeler mutant mice (P60). (a) Quantification of immunostaining shows that HMGN3 highly expressing astrocytes are enriched in the upper layer in control mice (P60). (b) Quantification of immunostaining shows that the ULA-enriched expression of HMGN3 protein was disrupted in reeler mutant mice (P60). Relative distance from the corpus callosum (CC); layer I, 0.9-1; layer II/III, 0.7-0.9; layer IV, 0.5-0.7; layer V, 0.2-0.5; layer VI, 0-0.2 as an approximate position in the cortical layers.

## Conclusion

Taken together, these results based on morphological and molecular features provide the first evidence showing that neocortical astrocytes are layer-specifically heterogeneous even among the protoplasmic astrocytes in layers II/III-VI that have long been considered as a non-diverse population. Intriguingly, neocortical astrocytes in distinct layers differentially express various genes involved in synaptic regulation, morphogenesis, and metabolism. Expression differences of these functional genes might lead to different functions and/or interactions with neighbouring neurons and synapses, providing a hint about functional heterogeneity of astrocytes in the mouse neocortex. Our findings open up avenues for future study, such as the functions and properties of astrocyte subtypes, and the subtype-determining mechanisms that generate this astrocyte diversity in the neocortex and other regions of the CNS.

[1] Motoshi Nagao, Darin Lanjakornsiripan, et al. (2014) High Mobility Group Nucleosome-Binding Family Proteins Promote Astrocyte Differentiation of Neural Precursor Cells. *Stem Cells.* 32(11):2983-97.