博士学位論文審査要旨

２０２３年1月３０日

論文題目：　The Role of $[Ca^{2+}]_i$ Fluctuation in Cortical Neural Progenitor Cells
（神経幹細胞における細胞内カルシウム変動の役割）

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要　　旨：

本学位論文では、胎児期における神経幹細胞の制御メカニズムについて検討を行っている。哺乳類において、大脳皮質は適応的行動を形成する上で重要である。胎児期には、神経幹細胞から適切な数の神経細胞が生産され、大脳皮質特有の層構造が形成される。このシステムが正しく働くには、細胞分裂によって適切な数の神経幹細胞が維持され、かつ適切なタイミングで自己再生型の幹細胞から神経細胞産生型の幹細胞へと分化する必要がある。この幹細胞の分化状態の制御メカニズムは十分に理解されていない。本学位論文では、細胞内セカンドメッセンジャーであるカルシウムイオン（Ca^{2+}）に着目し研究を行なっている。神経幹細胞では細胞内 Ca^{2+}濃度が自発的に変動することが知られている。申請者はこの Ca^{2+}濃度変動が神経幹細胞の分化制御に働くとの仮説を立脚し、その検証を試みている。

まず、大脳皮質形成期のマウス胎児から急性スライス標本を作成し、自己再生型の幹細胞が多い胎生１１日、神経細胞産生型の幹細胞が多い胎生１２日以降などで、Ca^{2+}濃度変動に変化があるかどうかを検証した。その結果、胎生１２日以降に比較的速い Ca^{2+}濃度変動を呈する細胞が増えることが分かり、また、急性分散細胞標本においても Pax6 陽性の自己再生型幹細胞よりも Pax6/Btg2 二重陽性の神経細胞産生型幹細胞がこのような速い Ca^{2+}濃度変動を呈するようになることを見出した。さらに、この Ca^{2+}濃度変動が T 型カルシウムチャネルによる細胞外からの流入によることを薬理学実験や遺伝的実験で明らかにし、これらのツールを用いて T 型カルシウムチャネル抑制すると、培養実験において神経幹細胞の増殖が増大し、逆に神経細胞の産生が抑制されることを示唆する結果を得ている。また胎児においても、T 型カルシウムチャネル発現を抑制することで、産生される神経細胞の数が有意に減少することを見出した。これらの結果から申請者は、T 型カルシウムチャネルを介した Ca^{2+}濃度変動が神経幹細胞の分化を制御すると結論している。これらの知見は、発達期の正常な大脳皮質形成の理解に貢献するのみならず、成体における神経新生のメカニズム理解にも重要であると考えられる。

口頭試問では、英語による適切な研究発表を行った。質疑応答では論文についての問題点がいくつか指摘されたが、それに対して満足のできる回答・討論を行い、学位論文の defense を行うことができたと判断できる。

よって、本論文は、博士（理学）（同志社大学）の学位を授与するにふさわしいものであると認める。

総合試験結果の要旨

２０２３年１月３０日

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要　　旨：

博士学位候補者の PUTU ADI ANDHIKA RHADITYA 氏に対して、2023 年 1 月 27 日午後 5 時より総合試験を行った。

対象となる学位研究は、胎生期における神経幹細胞の分化制御における細胞内カルシウム変動の役割を検討するものである。そこで総合試験では主に、神経生物学、神経生理学、統計学の基礎および専門的な知識についての試問を行なった。なお、語学試験については、総合試験の前に行った公開口頭試問を英語で行い、学位論文の内容を明確に説明することができた。研究に必要な外国語に通じていることが認められたため、総合試験では免除とした。

PUTU ADI ANDHIKA RHADITYA 氏は、審査委員の質問について適切に答え、不明な点に関しては十分に論理的な考察を行った。関連分野の基礎知識は十分に備わっていると判断した。専門的な知識については、まだまだ不十分な点も認められるが、概して満足のできるレベルにあると判断した。

以上を踏まえて、審査委員一同の協議の結果、総合試験の結果は合格であると認める。

博 士 学 位 論 文 要 旨

論 文 題 目 ： The Role of $[Ca^{2+}]_i$ Fluctuation in Cortical Neural Progenitor Cells（神経幹細胞における細胞内カルシウム変動の役割）

氏　　名 ： PUTU ADI ANDHIKA RHADITYA

要　　旨 ：

Neural progenitor cells (NPC) are stem cells that can differentiate into all types of neurons and have an essential role in the development of central nervous system. During cortical development at less than day 11 (E11), NPCs proliferate by self-renewing to increase their number in the ventricular zone (VZ). However, at E11, some NPCs gradually differentiate into neuron-generating NPCs to produce neurons. Furthermore, at E14-E16, most NPCs are already differentiated into neuron-generating NPCs. Differentiation into neuron-generating NPCs is a process by which self-renewing NPCs lose their potential "stemness" or reduced self-replication ability by generating differentiated cells. Neuronal differentiation is a complex process by which NPC acquires electrophysiological and morphological characteristics specific to neurons to form circuits, and later these cells can process incoming information. However, the mechanisms controlling the differentiation of undifferentiated self-renewing NPCs into neuron-generating NPCs still need to be discovered.

Calcium ion (Ca^{2+}) is one candidate that regulates NPC development. Ca^{2+} is an intracellular second messenger that regulates many cellular processes, including embryonic events from fertilization to organogenesis. Interestingly, the spontaneous $[Ca^{2+}]_i$ fluctuation is frequently observed during cortical development and is known to regulate NPC development, such as neural induction, the proliferation of NPCs, migration, and specification of immature neurons. However, little is known about the role of $[Ca^{2+}]_i$ fluctuations in NPCs during early developmental stages, especially during their differentiation from self-renewing neuron-generating NPCs and immature neurons remains to be determined. Thus, this study aimed to investigate whether $[Ca^{2+}]_i$ fluctuations in NPCs are involved in regulating their differentiation.

Using the live-imaging of calcium indicator Fluo-4 on embryonic-derived cortical slices and suspended cells, we examined temporal changes in the pattern of $[Ca^{2+}]_i$ fluctuations in VZ cells from E11 to

E16. Firstly, we classified the pattern of the Fluo-4 signal into two groups according to the presence or absence of this intermittent $[Ca^{2+}]_i$ transient. The intermittent $[Ca^{2+}]_i$ transient is an event where the intensity of Fluo4 increases beyond half of the baseline of the cell itself in such a short time (less than 3 minutes). As a result, we found that at later stage E16, many cells show $[Ca^{2+}]_i$ fluctuations with intermittent $[Ca^{2+}]_i$ transients compared to the early stage E12. Furthermore, this increase in the number of intermittent $[Ca^{2+}]_i$ transients is accompanied by a decrease in the level of $[Ca^{2+}]_i$. Although our observations suggested developmental changes of $[Ca^{2+}]_i$ fluctuations in VZ cells, VZ contains many different cell types, such as NPCs, intermediate progenitor cells and immature neurons. Therefore, to understand the relationship between cell type and of $[Ca^{2+}]_i$ fluctuations, we identified each cell type by immunohistochemistry of the cells isolated from the embryonic cortex after recording the Fluo-4 signal. First, we focused on the Fluo-4 signal fluctuation pattern in Pax6-expressing cells, because Pax6 is a transcription factor that controls the identity of NPCs and plays essential roles in self-renewing and differentiation in the developing NPCs. As a result, we found similar results when we examined Pax6-positive NPCs in suspended cells. From E11 to E16, the number of intermittent $[Ca^{2+}]_i$ transients in Pax6-positive NPCs seemed to increase gradually, while the $[Ca^{2+}]_i$ gradually decreased, which is consistent with the imaging results of brain slices. The generation of intermittent $[Ca^{2+}]_i$ transient may correlate with the differentiation of Pax6-positive cells into immature neurons.

At the early stage, most of the Pax6-positive cells exhibited the $[Ca^{2+}]_i$ fluctuation without intermittent $[Ca^{2+}]_i$ transient. However, many Pax6-positive cells with intermittent $[Ca^{2+}]_i$ transients at a later stage (E16). Based on these findings, we decided to examine whether immature neurons (Tuj1-positive cells) exhibit more intermittent $[Ca^{2+}]_i$ transients. To examine the pattern of the Fluo-4 signal of Tuj1-positive cells, we identified Tuj1-positive cells after imaging experiments. Interestingly, fewer Pax6-positive NPCs exhibited $[Ca^{2+}]_i$ transient; in contrast, many immature neurons show it. Because many Tuj1-positive immature neurons exhibited intermittent $[Ca^{2+}]_i$ transient, compared to Pax6-positive cells, these results suggest that the onset of the intermittent $[Ca^{2+}]_i$ transient may be a hallmark of differentiation.

Btg2 is an anti-proliferative gene that represses the progression of the cell cycle in NPCs and causes differentiation into neurons. In the previous result, we found that Tuj1-positive immature neurons exhibited more intermittent $[Ca^{2+}]_i$ transient than Pax6-positive NPCs. Therefore, we investigated whether the onset of intermittent $[Ca^{2+}]_i$ transients correlates with the initiation of Btg2 expression in NPCs. Although at E14, we found an insignificant difference in the number and intensity of intermittent $[Ca^{2+}]_i$ transients between Pax6-positive Btg2-negative self-renewing NPCs and Pax6 Btg2 double-positive neuron-generating NPCs;

however, at E11, Pax6 Btg2 double-positive neuron-generating NPCs showed more intermittent $[Ca^{2+}]_i$ transient than in Pax6-positive Btg2-negative self-renewing NPCs. Together, these results suggest that the change in the pattern of $[Ca^{2+}]_i$ fluctuation correlate to their differentiation.

To understand the molecular mechanisms of $[Ca^{2+}]_i$ fluctuation in NPCs, we performed calcium imaging experiments on embryonic cortex slices treated with Thapsigargin (internal Ca^{2+} storage blocker). Then we also eliminated extracellular Ca^{2+} using EGTA to check the origin of the Ca^{2+} source. We observed that the Fluo-4 signal was significantly reduced after we eliminated extracellular Ca^{2+} by treatment EGTA but not changed after blocking internal Ca^{2+} storage. These results indicate that the Ca^{2+} entry mediates the observed fluctuation of $[Ca^{2+}]_i$. To identify the Ca^{2+} channel, we examined the effects of several inhibitors for Ca^{2+} channels. Treatment with an inhibitor of L-type Ca^{2+} channels had a slight inhibitory effect on the $[Ca^{2+}]_i$ fluctuation. In addition, an inhibitor of both T-type and L-type channels also slightly reduced the number of cells exhibiting the $[Ca^{2+}]_i$ fluctuations with intermittent $[Ca^{2+}]_i$ transient. In contrast, a specific inhibitor for the T-type calcium channel in Cav3.1 and Cav3.2 significantly blocked the $[Ca^{2+}]_i$ fluctuation, suggesting that the $[Ca^{2+}]_i$ fluctuations observed in the NPCs were mediated by T-type calcium channels.

Finally, we examined the effect of blockage T-type calcium channels on NPC differentiation in the living tissue; we inhibited the expression of the T-type calcium channel in NPCs in vivo using the RNAi technique. There are three subtypes of T-type calcium channels, Cav3.1, Cav3.2, and Cav3.3. It has been reported that many Cav3.1 is expressed in NPCs. To inhibit Cav3.1 expression in developing cortical NPCs, we introduced an RNAi expression vector for Cav3.1 and a GFP expression vector into cortical VZ cells using in-utero electroporation. At 24 h after electroporation, we examined whether the GFP-positive cells expressed Pax6 or Tbr1. We examined Tbr1-positive cells as immature neurons. As a result, among cells with Cav3.1 knockdown, the proportion of Pax6-positive cells was significantly more than that of Tbr1-positive cells. These results suggested that Cav3.1 knockdown maintains NPCs as a Pax6-positive cell. In contrast, in cells transfected with the control vector, the proportion of Pax6-positive cells and Tbr1-positive cells was almost same. Together, these results suggest that $[Ca^{2+}]_i$ fluctuation through T-type calcium channels is required to differentiate from the undifferentiated self-renewing NPCs into the neuron-generating NPCs.

Understanding the signaling molecules involved in $[Ca^{2+}]_i$ fluctuations in NPCs is critical to elucidate the mechanisms by which NPCs can differentiate into neurons during the development of the mammalian brain. Furthermore, this knowledge may also help to understand diseases caused by an abnormal balance between self-renewal and differentiation of NPC. In this study, we provide a model in which the onset

of intermittent $[Ca^{2+}]_i$ transients mediated by Cav3.1 T-type calcium channels in self-renewing NPCs correlates with their differentiation into neuron-generating NPCs. However, several questions still need to be answered related to intermittent $[Ca^{2+}]_i$ transients, including the causes of intermittent $[Ca^{2+}]_i$ transients and what is transcription factors that activated by intermittent $[Ca^{2+}]_i$ transients. Therefore, further experiments using agonists/promoters for NPCs differentiation need to be carried out to observe changes in $[Ca^{2+}]_i$ fluctuation patterns and gene expression profiles during these proses.