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学位記番号	総研大甲第867号
学位授与の日付	平成１７年３月２４日
学位授与の要件	生命科学研究科　遺伝学専攻 学位規則第６条第１項該当
学位論文題目	Intrinsic sub-axonal patterning in Drosophila Neurons: Compartment boundaries in axons regulate the localization of Robo receptors
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論文内容の要旨

During the development of the nervous system, neurons extend their axons over a long distance to their targets with the assistance of guidance cues and guidance receptors. Although we have detailed knowledge about the variety of these guidance molecules, little is known about how the distributions of the guidance molecules are controlled in space and time. Immunohistochemical studies have revealed that the spatial distribution of axon guidance receptors is often regulated at sub-cellular levels, or rather at sub-axonal levels. For example, in the *Drosophila* ventral nerve cords, a repulsive guidance receptor Roundabout and its family members (Robo2 and Robo3) are specifically expressed in the longitudinal axon tracts, which indicates that Robo receptors are localized to the distal segment of the axons but excluded from the proximal segment of the same axons. This restricted expression of Robo within axons is largely conserved from fruit flies to mammals. Moreover, recent findings that receptors can influence the distribution of guidance cues underscore the importance of the regulated expression of guidance receptors.

A fundamental question about such sub-axonal localization of molecules is whether the localization is dependent on extrinsic signals such as cell-cell contacts, or is due to intrinsic properties of neurons. Although it has long been known that neurons possess an intrinsic ability to acquire axonal and somatodendritic domains, the possibility that neurons have an intrinsic ability to generate sub-axonal domains remains to be explored. Another question is how the localization of transmembrane molecules in certain region of the axonal membrane is established and maintained. Molecules should first be asymmetrically delivered to the axonal membrane, and then the asymmetric distribution of the molecules should be retained despite the fact that the axonal membrane is continuous and fluid.

To distinguish between the contributions of extrinsic factors and intrinsic properties on the development of the sub-axonal localization of molecules, I used a low-density primary culture system of *Drosophila* neurons where neurons extend their axons without cell-cell contacts. I reasoned that if neurons have an intrinsic property to create sub-axonal localization of molecules, localization of Robo receptors could be seen in the cultured neurons. When cultured neurons were immunostained for Robo proteins, cultured neurons exhibited uniform distribution of Robo along their axons, suggesting that Robo may require extrinsic cues for its localization. In contrast, Robo2 and Robo3 were localized to the distal segment of the axons even in cultured neurons. This result indicates that neurons possess an intrinsic property to generate the localization of Robo2 and Robo3.

I next asked how the localization of Robo3 in the distal axon segment is established during the course of axonal elongation. One possibility is that molecules are delivered only to the growing tip of the axon, and the localization pattern along the axon is determined by the temporal pattern of the ON and OFF of the delivery. To examine the course of localization, I performed time-lapse recordings of growing neurons that express Robo3-EGFP fusion proteins. When axons just started to extend, Robo3-EGFP was detected only in cell bodies. At around 8

hrs from the onset of axonal elongation, strong GFP signals appeared at the distal segment of the axons. Localization of Robo3-EGFP at the distal axon segment occurred after axonal elongation but not together with axonal elongation. In addition, when the fluorescent signal of Robo3-EGFP in the distal axon segment was eliminated by intense 488 nm laser illumination, Robo3-EGFP signal reappeared to the distal axon segment without further axonal elongation (13% recovery in 40 minutes). Thus, it is likely that the localization of molecules and axonal elongation are uncoupled processes.

Next, I examined how the localization of transmembrane molecules such as Robo receptors in the axonal membrane is maintained. One possibility is that molecules are immobilized along the axon by being anchored to the membrane cytoskeleton and therefore the localization patterns can be retained. To test this possibility, I assessed the dynamics of molecules that are localized to the distal segment of the axon using a fluorescence recovery after photobleaching (FRAP) method. In FRAP experiments, a discrete region of the cell is photobleached, and then the recovery of fluorescence over time is measured, which gives information about the mobility of molecules. When FRAP analysis of Robo3-EGFP was performed in the distal axon segment, fluorescent signals recovered to the photobleached area in an exponential manner, suggesting that Robo3-EGFP is diffusible along the axon. Thus, anchoring model seems to be insufficient to explain the persisted localization of Robo3 in the axon.

Given that Robo3-EGFP is mobile in the axonal membrane, how is Robo3-EGFP kept from uniformly distributing along the axon? One possibility is that there exists a diffusion barrier between the distal and proximal segments of the axon, and that the barrier prevents Robo3-EGFP from entering into the proximal segment. I reasoned that if there exists a diffusion barrier at the boundary between the distal and proximal segments (Robo3-EGFP localization defines the distal segment), then the mobility of Robo-EGFP across the boundary could also be restricted by the diffusion barrier. In this case, FRAP experiments should result in that fluorescent signals recovering more slowly from the side close to the boundary compared to the side away from the boundary. Conversely, if there is no diffusion barrier at the boundary, fluorescent signals should recover equally from both ends of the photobleached area. When the proximal part of the axon from the boundary was photobleached, less Robo-EGFP signal recovered from the side close to the boundary compared to the side close to the cell body. Thus, it is likely that the mobility of molecules across the boundary of segments is restricted by a diffusion barrier mechanism.

Finally, I tested whether this diffusion barrier acts specifically to Robo family of receptors or acts generally to transmembrane proteins. The mobility of CD8-GFP, a transmembrane molecule that has no similarity to Robo receptors, was tested by FRAP experiments. The mobility of CD8-GFP across the boundary was also restricted. This result suggests that the boundary between the proximal and distal compartments acts as a general diffusion barrier to transmembrane proteins.

Using a primary cell culture system of *Drosophila*, here I have demonstrated that neurons

have an intrinsic property to create the sub-axonal localization of Robo2 and Robo3. FRAP experiments revealed that the mobility of transmembrane proteins was restricted at the boundary between the segments. I propose that neurons possess an intrinsic property to compartmentalize their axons by a diffusion barrier mechanism, and that the compartment boundary regulates the localization of Robo2 and Robo3. The result that Robo was uniformly distributed along the axons in cultured neurons suggests that neurons might use extrinsic factor(s) to generate the localization of Robo. It would be also interesting to examine whether the localization of Robo2 and Robo3 could be further adjusted by extrinsic factors. Robo receptors would provide insights into how intrinsic properties of neurons and extrinsic factors participate in regulating the distribution of guidance receptors.

論文の審査結果の要旨

本研究は、神経回路網のパターン形成に関し、神経細胞内に拡散障壁があり、神経細胞が自律的に、軸索ガイダンス分子の局在パターンを軸索内に形成し得る事を見い出した。

神経軸索の投射を制御する分子は軸索ガイダンス分子と呼ばれ、神経回路網の形成において必須の役割を果たしている。軸索ガイダンス分子の受容体は、軸索の特定の領域に限局して分布することが多いが、軸索内に分子を局在させる制御機構は未知である。1回膜貫通型のRoboファミリー(Robo, Robo2, Robo3)は軸索ガイダンス分子の受容体で、生体内において軸索の遠位端部分に局在する事が示されていた。しかし、他の細胞との接触で軸索内局在が形成されるのか、それとも神経細胞の自律的な性質によって形成されてるのか、不明であった。ショウジョウバエの初代培養系を用いることにより、他の細胞との接触のない条件下で神経細胞を成長させ、軸索内でのRobo受容体の分布を免疫染色法とGFP融合タンパク質の二つの方法を用いて観察した。

Robo は、培養条件下では軸索全体に均一に分布した。このことは、Robo の軸索内局在には他の細胞との接触が必要である可能性を示唆する。一方、Robo2とRobo3は培養条件下においても、軸索の遠位端にのみ再現性よく局在することが見つかった。BP102 モノクローナル抗体は、Robo3と相補的なパターンで軸索の近位端のみ染色した。これらの知見は、神経細胞が自律的に、軸索内に分子の局在を形成する能力を有していることを示している。

軸索内での分子の局在はどのように形成・維持されているか、Fluorescence Recovery After Photobleaching (FRAP)法を用いて、軸索での Robo 受容体の動態を調べた。その結果、これらの受容体が軸索上で拡散しうることが明らかになった。したがって、Robo2 や Robo3 の局在を形成維持するためには、軸索の特定の場所に分子の拡散を妨げる障壁がある、分子を局所的に分解する、等のいずれかのメカニズムが必要であると考えられた。この疑問に答えるべく、RoboとCD8-GFP(どちらも軸索全体に分布する)の、Robo3局在領域の境界(遠位端と近位端の境界)における動態を FRAP 法によって解析した。その結果、局在境界においてRoboとCD8-GFPの拡散が抑制されていることを見いだした。これは、軸索の特定の領域に拡散障壁が存在することを示している。

BP102 と Robo3 の局在の境界は、拡散障壁の領域において一致していた。このことは、拡散障壁が分子の局在を維持する共通のメカニズムである可能性を提起する。また、拡散障壁のある局在境界領域に特異的に局在する分子の存在も見いだしている。

以上、勝木は、神経細胞が自律的に軸索内に分子の局在パターンを形成する能力を有していること、複数分子の局在境界領域に共通の拡散障壁が存在することを見出した。この発見を基に、神経の軸索は拡散障壁によって複数の異なる区画に分割されており、それぞれの区画が分子の局在を制御する基本構造として機能している、という分子機構モデルを提出している。

高い独自性のもと、重要な知見を得、新しい分子機構モデルへと展開しており、関連分野への寄与は大きい。審査委員会は、本研究が学位授与の要件を十分に満たすものと判断した。

公開発表会とそれに続く非公開の審査委員会の質疑応答と、提出された論文を基に審議し、専門および関連分野での知識・理解・考察力に優れており、学位にふさわしいと判断した。博士論文の英文、公開発表の英語発表から、英語に関して優れた実力を有していると判断した。