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論文内容の要旨

During embryonic nervous system development, individual neurons extend axons, long and thin processes that connect to distant cells, to establish precise neuronal networks. The growth cone, a highly motile amoeboid structure at the tip of the elongating axon, navigates the axon along the correct path by sensing concentration gradients of axon guidance cues presented in the extracellular local environment (Tessier-Lavigne and Goodman, 1996). Accumulating evidence indicate that intracellular second messengers, such as Ca^{2+} and cyclic AMP (cAMP), play crucial roles in the control of guidance cue-induced growth cone behaviors (Tojima et al., 2011). The guidance cue gradients evoke asymmetric increase in cytosolic Ca^{2+} concentration, with higher Ca^{2+} on the side of the growth cone facing the source of the cues, regardless of whether the cues are attractive or repulsive. Such asymmetric Ca^{2+} signals are necessary and sufficient to trigger both attractive and repulsive growth cone turning with respect to the cues. Importantly, the distinction between attractive and repulsive Ca^{2+} signals is the occurrence of Ca^{2+}-induced Ca^{2+} release (CICR) from the endoplasmic reticulum (ER) Ca^{2+} store through ryanodine receptors (RyRs): Ca^{2+} influx through plasma membrane Ca^{2+} channels alone triggers growth cone repulsion, whereas the Ca^{2+} influx together with CICR triggers attraction (Ooashi et al., 2005). The occurrence of CICR can be controlled by cAMP signals: higher cAMP signals push the RyRs to the active state, allowing CICR, whereas lower cAMP signals inactivate the RyRs (Ooashi et al., 2005). In contrast to cAMP, however, less is known about the role of cyclic GMP (cGMP) in growth cone behaviors.

In the first part of this thesis (**Chapter 1**), I examine the role of cGMP and its upstream activator, nitric oxide (NO), in the regulation of CICR to control the directional polarity of growth cone guidance (Tojima et al., 2009). Using cultured embryonic chicken dorsal root ganglion neurons, I show that activation of the NO-cGMP pathway abolishes CICR and converts

Ca^{2+}-mediated growth cone attraction into repulsion. On the other hand, inhibition of the NO-cGMP pathway allows CICR and converts growth cone repulsion into attraction. Importantly, the NO-cGMP pathway counteracts the effect of cAMP on growth cone guidance. I also show that extracellular substrates affect the polarity of growth cone guidance via modulating cAMP and cGMP levels in an opposite manner. These results demonstrate a novel second messenger network that dictates bidirectional growth cone behaviors in response to the same guidance cues.

What intracellular mechanisms act downstream of second messengers to drive growth cone guidance? It is well known that asymmetric reorganization of cytoskeletal and adhesion components across the growth cone plays a critical role: the growth cone turns toward or away from the side with stabilized or destabilized cytoskeletons/adhesion, respectively (Lowery and Van Vactor, 2009; Myers et al., 2011). In addition to these well-established mechanisms, it is possible that membrane trafficking system controls growth cone guidance. Recently, our laboratory reported that attractive Ca^{2+} signals, generated on one side of the growth cone, facilitate microtubule-dependent centrifugal transport of intracellular vesicles toward the leading edge and subsequent vesicle-associated membrane-protein 2 (VAMP2)-mediated exocytosis on the side with Ca^{2+} elevation (Tojima et al., 2007). Furthermore, this asymmetric exocytosis is necessary for Ca^{2+}-dependent growth cone attraction. However, it remains unknown what mechanisms drive growth cone repulsion downstream of Ca^{2+} signals.

In the second part of this thesis (**Chapter 2**), I examine the role of endocytosis in growth cone guidance (Tojima et al., 2010). Using total internal reflection fluorescence microscopy, I show that repulsive, but not attractive, Ca^{2+} signals induce an asymmetry in the frequency of clathrin-mediated endocytosis across the growth cone. I also show that pharmacologic or genetic inhibition of clathrin-mediated endocytosis abolishes Ca^{2+}-dependent growth cone repulsion, but not attraction. My results, along with our previous report (Tojima et al., 2007), demonstrate that growth cone attraction and repulsion are driven by opposite membrane trafficking events: plasma membrane addition and removal, respectively (**a-c** in **Figure**).

In the last part of this thesis (**Chapter 3**), I further examine the antagonistic actions between exocytosis and endocytosis in determining the polarity of growth cone guidance (Tojima et al., 2014) (**d** in **Figure**). I show that growth cone turning direction depends on imbalance between VAMP2-mediated exocytosis and clathrin-mediated endocytosis on one side of the growth cone. I also identify the signaling pathways that regulate such localized imbalance between exocytosis and endocytosis downstream of Ca^{2+} signals. Repulsive Ca^{2+} signals facilitate clathrin-mediated endocytosis through the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin and a 90-kD splice variant of phosphatidylinositol-4-phosphate 5-kinase type-1γ (PIPKIγ90). In contrast, attractive Ca^{2+} signals facilitate exocytosis but suppress endocytosis through Ca^{2+}/calmodulin-dependent protein kinase II and cyclin-dependent kinase 5 that can inactivate PIPKIγ90. My results illustrating the antagonistic effects between exocytosis and endocytosis imply that endocytic and exocytic membrane vesicles carry functionally similar cargo molecules such as cytoskeletal and adhesion components.

Taken altogether, my research establishes a novel mechanistic concept for growth cone guidance, in which polarized membrane trafficking acts, downstream of second messengers, as an instructive mechanism to spatially localize cytoskeletal and adhesion components (Itofusa and

Kamiguchi, 2011). This conceptual advance on growth cone biology will contribute to our better understanding of the mechanisms of human disorders with aberrant axon connectivity and to technological innovations for rewiring of axons to their appropriate targets following injury to the adult central nervous system.

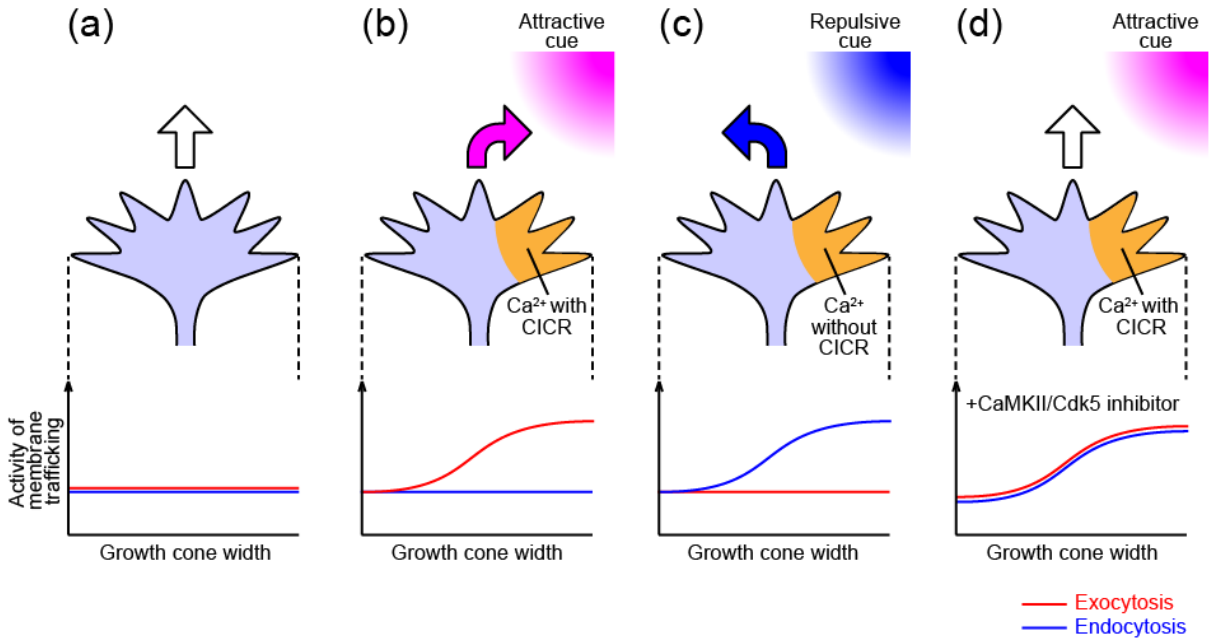

Figure. Localized imbalance between exocytosis and endocytosis steers the growth cone
(a) In the absence of guidance cues, the growth cone migrates straight because the activities of exocytosis (red line) and endocytosis (blue line) are symmetric across the growth cone width. **(b)** The attractive cue (pink) evokes Ca^{2+} influx together with CICR and thereby activates exocytosis on the side of the growth cone facing the cue, while endocytosis remains symmetric. Such localized predominance of exocytosis over endocytosis causes attractive turning toward the cue. **(c)** The repulsive cue (blue) evokes Ca^{2+} influx without CICR and thereby causes endocytosis predominance on the side facing the cue, resulting in repulsive growth cone turning. **(d)** Application of CaMKII or Cdk5 inhibitor leads to balanced activation of both exocytosis and endocytosis on the side facing the attractive cue. Such asymmetries in exocytosis and endocytosis with the same polarities cause straight migration even if the cue is attractive.

Publications

*Tojima T, ***Itofusa R**, Kamiguchi H (2009) The nitric oxide-cGMP pathway controls the directional polarity of growth cone guidance via modulating cytosolic Ca^{2+} signals. Journal of Neuroscience 29:7886-7897. (*These authors contributed equally; Thomson Reuters impact factor

2013: 6.747) **[Chapter 1 in this thesis]**

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*Tojima T, ***Itofusa R**, Kamiguchi H (2014) Steering neuronal growth cones by shifting the imbalance between exocytosis and endocytosis. Journal of Neuroscience 34:7165-7178. (*These authors contributed equally; Thomson Reuters impact factor 2013: 6.747) **[Chapter 3 in this thesis]**

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審査結果の要旨

神経突起が伸長する際､成長円錐は環境中の非対称な刺激を感知して伸長方向を決定(成長円錐ガイダンス）するが、この伸長方向の決定には成長円錐内での Ca^{2+}濃度の非対称な上昇が重要な役割を担っており、Ca^{2+}濃度上昇が形質膜の Ca^{2+}チャネルからの流入による場合には刺激から反発される方向に、Ca^{2+}濃度上昇が Ca^{2+}誘発性 Ca^{2+}放出を伴う場合には刺激に誘引される方向に向かうことがこれまでの研究で明らかにされている．本論文は軸索ガイダンスにおける Ca^{2+}誘発性 Ca^{2+}放出の制御における cGMP 系情報伝達の関与及び、細胞内 Ca^{2+}濃度上昇の下流における endocytosis・exocytosis の重要性を作業仮説として、その検討をおこなったものである．鶏卵胚後根神経節細胞の培養標本を実験材料とし、Ca^{2+}イメージング法による Ca^{2+}濃度変化の観察や全反射照明蛍光顕微鏡による endocytosis・exocytosis の可視化などの手法を駆使してこれらの問題を検討した結果、1)細胞内 Ca^{2+}濃度上昇に依存する神経成長円錐伸長の方向制御が NO-cGMP 系による制御を受けていること、2)反発性の方向制御がクラスリンの関与する endocytosis の非対称性によってもたらされること、3)細胞内 Ca^{2+}濃度上昇の下流で引き起こされる exocytosis と endocytosis との局所的な不均衡が方向転換をもたらすこと等を明らかにした.

従来、軸索の前後軸での伸長・後退の制御については、細胞接着分子と線維性アクチンの結合に加え、膜小胞の軸索輸送及び endocytosis・exocytosis の協調による極性膜 trafficking が重要な役割を果たしているという描像が得られていた．本研究及び本研究に先行する一連の研究は、軸索の方向決定においても極性膜 trafficking が重要な役割を果たしていることを示し、そのシグナル経路を解明したものであり、極性膜 trafficking の成長円錐ガイダンスにおける機能についての総合的見解は、申請者を筆頭著者とする総説に公表されている．今後、膜 trafficking によって運ばれる膜受容体や細胞接着分子の解明、細胞骨格を形成する分子の重合・脱重合など軸索の伸長・後退を制御する機構との関連の解明、さらには標的に到達した後のシナプス形成過程との関連の解明など、多くの重要な問題への展開が期待される.

本研究の目的は明確であり、作業仮説の検証に適した手法と実験計画によって研究を実施し、観察データに基づいたしっかりとした解析から新規かつ独自な結論を導いている．本研究の成果は軸索ガイダンスの初期過程を解明したものであって、内外の研究グループによる従来の研究成果とあいまって、生命科学の進歩に大きく貢献する十分な学術的意義が認められる．将来的に再生医療やバイオ素子開発等に貢献することも十二分に期待できる研究成果である．予備審査及び本審査における研究内容の提示は明快であり、論文内容及び関連分野・学問に関する試問に対する受け答えは、深い知識と理解に裏付けられた高い学力を示すとともに、主体的・意欲的に研究課題に取り組んできたことを示すものであった.

以上の事から、審査委員は全員一致で本学位論文を博士（生命科学）の学位に値するものと認める.