ウェブサイト公開用

（様式 10）
（海外特別研究員事業）

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海 外 特 別 研 究 員 最 終 報 告 書

独立行政法人 日本学術振興会 理事長　殿

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氏　　名

（氏名は必ず自署すること）

海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1. 用務地（派遣先国名）用務地：　Baltimore　（国名：United States of America）

2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。
シナプス小胞膜分断装置の超高速形成・収縮機構の解析を基盤とした膜分断の普遍性解明

3. 派遣期間：平成　28 年　4 月　1 日　～　平成　30　年　3 月　31　日

4. 受入機関名及び部局名
Johns Hopkins University school of medicine, department of cell biology

5. 所期の目的の遂行状況及び成果…書式任意　**書式任意（A4 判相当 3 ページ以上、英語で記入も可）**
（研究・調査実施状況及びその成果の発表・関係学会への参加状況等）
（注）「6. 研究発表」以降については様式 10－別紙 1～4 に記入の上、併せて提出すること。

My long-term objective is to understand the molecular mechanisms of synaptic vesicle regeneration. The vesicles are regenerated through two steps. First, fused synaptic vesicles after the arrival of action potential are retrieved from plasma membrane by endocytosis. Second, endocytic vesicle is delivered to endosome and synaptic vesicles are regenerated by budding off from the endosome. In general, membrane fission events are taken place ~5 sec – 1 min by the function of GTPase dynamin family member. However, those endocytic events during synaptic vesicle regeneration takes less than 100 ms, called ultrafast endocytosis, it is 50-600 times faster than the speed of traditional dynamin-based fission machinery. Thus, how does cell generate kinetics of this membrane fission is enigmatic.
To understand molecular mechanics of ultrafast membrane fission during synaptic vesicle regeneration, I proposed the following three aims.

A. Specific aims.
Aim 1. GTPase dynamin: I will test if dynamin isoforms are capable to pinche off the vesicle less than 100 ms.
Aim 2. Assembly kinetics of dynamin: I will determine the regulation of ultrafast kinetics of dynamin.
Aim 3. Proteomic analysis of endocytic pit: I will determine the molecular constituents of membrane fission rings in ultrafast endocytosis

B. Results.
Aim 1: Specific dynamin splice isoform which can generate ultrafast kinetics are identified

My first aim was to determine whether dynamin GTPase, a core component of membrane fission machinery in many endocytic pathways, is involved in ultrafast endocytosis. In mammalian brain tissue, dynamin-1 and dynamin-3 are highly expressed. Thus, I prepared primary cultured mouse hippocampal neuron from dynamin-1 or dynamin-3 single knockout or dynamin-1/-3 double knock out mice. These cultured neurons are expressing channel rhodopsin by virus transfection. After the optogenetic stimulation of the neuron, cells are frozen at the desired time point and observed by transmission electron microscopy. Endocytic vesicles are tracked by internalized ferritin particles.

In wild type neuron, endocytosis was taken place at 100ms after the stimulation. Then endocytic vesicle was delivered to synaptic endosome at 1 second. Synaptic vesicles are regenerated from the endosome at 3 to 5 seconds. In dynamin-1 single knockout or dyamin-1/-3 double knockout mice, I observed endocytic pits are stalled at the plasma membrane at 1 second. Accumulation of endocytic pits were still observed at 10 second. This endocytic defect was not observed in dynamin-3 single knockout and rescued by dynamin-1 overexpression. Thus, dynamin-1 is specifically involved in ultrafast endocytosis. During the analysis, I found that specific splicing variant of dynamin-1, terms dynamin-1xA (one of 10 splice isoforms) form drives ultrafast endocytosis. This xA variant has an extension at the C-terminus, which contains another binding site for endophilin. This binding is essential for ultrafast endocytosis. Thus, these analyses teased out the molecular basis of Dynamin-1 in ultrafast membrane scission at synapses. We are currently in the last stage of data collection, and the results will be submitted in peer-reviewed article.

Beyond what I proposed in this application, I found that both Dynamin-1 and Dynamin-3 are independently involved in endosomal budding. This finding opened up a new avenue in my research, studying the endosomal sorting mechanisms.

Aim 2. Assembly kinetics of dynamin: <u>Initial accumulation of dynamin is important ultrafast kinetics for membrane pinching-off.</u>

My second aim was to determine the regulation of dynamin assembly kinetics *in vivo*. Generally, dynamin molecules are recruited from cytosol one-by-one and assembled into functional ring-spiral structure at the membrane fission sites and then pinch off the membrane. This process takes 5 sec – 1 min. However, I found that dynamin-1xA form can pinch off membrane within 100 ms during synaptic vesicle endocytosis. To understand the molecular mechanism of the ultrafast membrane fission, I tested if phospho-regulation of dynamin PRD domain is involved. Within PRD domain, dephosphorylated two amino acids S774 and S778 are important for interaction with dynamin binding partners including syndapin and endophilin. These binding partners are important for the dynamin recruitment.

I introduced point mutations in the Dynamin-1xA S774 and S778 and making them either phosphomimetic or phospho-deficient. By phospho-deficient form of dynamin-1 xA expression in dynamin-1 knockout neuron, ultrafast endocytic defect is recovered. But phospho-mimetic form of dynamin-1xA expression, endocytosis is progressively slowed down. Consistently, syndapin knock down slowed down formation of internalized vesicles. Moreover, syndapin has function on early stage of the endocytic pit before pinching off. These analyses are indicting that dynamin-1xA molecules are reaccumulated on the endocytic pit or concentrated before membrane fission to pinch off the vesicle immediately after the formation of the endocytic pit. Combined with the result from Aim1, it will be submitted in peer-reviewed article.

Beyond what I proposed in this aim, I also found that local GTP concentration is a critical factor in assembly kinetics of dynamin in a heterologous system. Using cpYFP-based GTP sensor, GTP is locally enriched around dynamin-mediated endocytic sites and support dynamin assembly. This local enrichment is mediated by ATP-GTP converting enzyme, NME2. I also found a potential role of this enzyme in neural functions.

Aim 3. Proteomic analysis of endocytic pit: Endocytic structures are isolated from mouse hippocampal neuron by newly developed optogenetic tool MAGNETO.

My third aim was to determine the molecular constituents of membrane fission rings in ultrafast endocytosis. In my original application, I proposed to use dynamin inhibitor to enrich endocytic structures and perform pull-down assay using APEX2-biotin labeling. However, this approach also accumulated endocytic pits from other endocytic pathways such as clathrin-mediated endocytosis. I revised the original approach and developed a novel approach using magnetic nano-particles and optogenetics, termed as Magnetic endosomal trapping by optogenetics (MAGNETO). First, I established isolation of endocytic structures from synaptosomes by Ficoll-gradient centrifugation. Purity was determined by immunoblotting and immunofluorescence microscopy using specific antibody to endocytic structures, including synaptobrevin-2 and synaptotagmin-1. Next, I conjugated iron (II, III) oxide magnetic nanoparticles to antibodies against vesicle protein synaptotagmin-1. After the optogenetic stimulation, particles are taken up in to neurons through ultrafast endocytosis. Endocytic structures are then released from isolated synaptosomes by osmotic pressure. I observed isolated vesicles under the whole-mount negative staining electron microscopy, and 50 % vesicles contains magnetic nano-particles. These nano-particle positive vesicles are collected by the magnetic field. In combination with dynamin inhibitor, I’m currently trying to isolate the ultrafast endocytic pits from neurons and perform proteomic analysis.