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Neuronsefficiently meet the task of directing membrane proteins and lipids to their appropriate subcellular destinations under strict temporal and spatial control. The basic trafficking pathways involved in subcellular segregation of cellular components are generally believed to be conserved among eukaryotic cells. For example, mechanism underlying subcellular distribution of membrane proteins to apical and basal domains in epithelial cells might also be operating in neurons for differential localization of proteins to axon or somatodendritic region (Fig.1)(Dotti and Simons, 1990; Jareb and Banker, 1998). A diffusion barrier is also present in the two cell types to limit mixing of proteins and lipids across the compartments (Kobayashi et al., 1992). Although, some work has been done to understand the mechanism behind protein and lipid transport to distinct neuronal compartments, we lack a complete picture.

In *Drosophila*, Robo guidance receptors are one of the pioneers in directing axonal outgrowth. In the ventral nerve cord, Robo receptors are found on the longitudinal axon tracts but excluded from the commissures (Kidd et al., 1998; Rajagopalan et al., 2000b). Even at single cell level by mosaic analysis, I observed that Robo3 expression is confined to a specific neuronal compartment whereas the membrane marker protein myristoylated-mRFP distributes uniformly (Fig.S1). In primary neuronal culture, Robo2 and Robo3 proteins cell intrinsically reside exclusively in the compartment distal to the diffusion barrier that is about 25□m away from the cell body (Katsuki et al., 2009).

In the first part of my work, I characterized the previously identified diffusion barrier (Katsuki et al., 2009) that divides the *Drosophila* cultured neuron into two distinct compartments. In mammalian neurons a diffusion barrier at the axon initial segment is well characterized (Kobayashi et al., 1992; Nakada et al., 2003; Winckler et al., 1999). Ankyrin adaptor proteins form the basis of this barrier (Hedstrom et al., 2008; Jenkins and Bennett, 2001; Zhou et al., 1998). In *Drosophila*, ankyrins are present in two isoform, Ank1 and Ank2 (Dubreuil and Yu, 1994). Ank1 is ubiquitous but Ank2 is neuron specific. I examined both the GFP tagged ankyrin forms in *Drosophila* cultured neurons using UAS-Gal4 system. Ank2 GFP under a pan-neuronal driver did not accumulate specifically at the boundary of compartments (Fig.S2).However, Ank1 GFP when expressed in all the neurons under elavGal4 was distributed throughout the neuron but highly enriched at the compartment boundary after 24hrs of culture (Fig.2). I also investigated the approximate timing of barrier formation using Ank1 GFP localization as a marker. The barrier seems to appear between 12-18 hrs in culture as judged by clear Ank1 localization. It is not detected at 12 hrs but observed in half of the population by 18hrs (Fig.3). Given that ankyrins cross link membrane proteins to the spectrin-based cytoskeleton, they may be instrumental in the formation of a cytoplasmic filter even in *Drosophila* neurons

similar to their vertebrate counterparts (Dzhashiashvili et al., 2007; Song et al., 2009).

Next, I examined the axonal and presynaptic markers with respect to the Ank1 GFP marked barrier. For e.g., FasII, an axonal marker is also found in the same distal compartment as Robo3 (Fig.4D). Immunostaining for presynaptic proteins like synapsin, synaptotagmin (Fig.4A and B), cysteine string protein (Fig.S3A) are detected exclusively in the distal compartment. Other tagged proteins, like Derailed (Katsuki et al., 2009) and Apc2-GFP (Fig.S3B) localize to the proximal compartment in *Drosophila* cultured neurons. The localization patterns observed for the above mentioned proteins exactly matched their compartmentalized distribution to the axonal or somatodendritic region in mushroom body olfactory and antennal projection neurons (Rolls et al., 2007; Trunova et al., 2011). By definition dendrites exclude presynaptic markers (Sanchez-Soriano et al., 2005) and clearly in our cell culture system, presynaptic proteins are absent from the proximal compartment closer to soma. This suggested that the compartments distal and proximal with respect to the barrier are likely axonal and somatodendritic respectively.

Atleast in culture, Robo3 occupies the same neuronal compartment as synaptotagmin. At higher magnification, both Robo3-RFP and Synaptotagmin-GFP colocalize within synaptic bouton-like regions (presumptive presynaptic sites) of the axon or distal compartment (Fig.5B). This raises a possibility that FasII and Robo3, members of cell adhesion molecule family (CAM) may also play an instructive role in synapse formation or stability. Indeed, FasII has previously been suggested to have a role in synaptic stability (Thomas et al., 1997).

With the analogy to other polarized cells, neurons may operate similar trafficking mechanism for axonal localization of Robo receptors. Receptors may either reach the axon by direct targeting or by an indirect route that involves endocytosis but whatever pathway the receptor follows it must harbor localization signals within its sequence. Thus, transgenic flies carrying inducible GFP tagged deletion or chimeric Robo receptors were created and the proteins were expressed using UAS/Gal4 system. This work was initiated by Takeo Katsuki and Tony DeFalco who made some of the constructs that I would be referring to in this thesis.

Robo receptors (Robo1, 2 and 3) are single pass transmembrane proteins (Fig.6). All three Robo receptors harbor previously identified conserved cytoplasmic motifs (CC0-CC1) responsible for repulsive signalling. Deletion of intracellular region from Robo2 and Robo3 almost completely abolished their localization (~85% of neurons) (Katsuki, unpublished) (Fig.7). Adding intracellular of both Robo2 and Robo3 to CD8 (uniform membrane protein) was also found sufficient for axonal localization. Intracellular region of Robo3 was then further divided into N and C-terminal domains; where the C-terminal domain lacked CC0-CC1 motifs (Fig.6). Out of the two fragments, C-terminal (Robo3 1064-1342) was found sufficient for localization by adding it to CD8 (Fig.7 and 8B) (Tony DeFalco, unpublished). Interestingly, I found

that Robo3 even without the C-terminal domain (Robo3 1-1063) could also localize (Fig.7 & 8C). Next, I compared Robo2 and Robo3 C-terminal cytoplasmic sequences to map the intracellular motif (Fig.9) and found a five amino acid signal 'ITENK' that confers localization ability to the entire cytoplasmic region of both Robo2 and Robo3 receptor (Fig.10). However, mutating 'ITENK' in full length Robo3 had a partial effect (Fig.11). Less number of neurons showed axonal localization of mutated Robo3-GFP plus the intensity ratio in axon vs dendrite also reduced after mutation. This supported the idea that in addition to the intracellular region, extracellular also plays an important role in localization. The important region in the extracellular was then mapped to the Ig (Immunoglobulin) domains (Fig.12). Robo3 with ITENK mutation in the intracellular and its Ig domains swapped with Robo could no longer resides in the axonal compartment and appeared punctate along the length of neuron (Fig.13D). However, these puncta were not accessible by surface labeling (Fig.S6). This suggested either the receptor delivery is defective or its stability on the membrane. Thus the puncta can either be endosomes or undelivered axon transport vesicles.

Next, I examined if Robo3 receptors get delivered to the compartment via axonal transport. I treated the cells with Brefeldin A, a drug that fuses ER and golgi organelles blocking fast and slow anterograde vesicular trafficking. This treatment significantly trapped the newly synthesized the GFP tagged receptor in the soma as compared to the control and resulted in loss of receptor delivery (Fig.14). To gain more insight into the possible localization mechanisms (Fig.15), I investigated delivery of Robo3and the two localization domainsby inducing receptor expression after the ankyrin-based barrier is formedin 'mature differentiated' neurons. Newly synthesized proteins in mature neurons were followed by time lapse imaging(Fig.16). Robo3 and Robo31064-1342 were detected first in the axon (Fig.17 and 18). On the other hand, Robo3 1-1063 was targeted randomly and even when it appeared in the axon at initial time points it was not stabilized and finally appeared more in the dendritic compartment (Fig.19). This suggested that C-terminal intracellular region of Robo3 possessed a dominant axon targeting property that might be enhanced in the presence of extracellular region. This result was also reproducible in 'mature' mushroom body (MB) neurons where Robo3 1-1063 was found exclusively in the dendritic region and not in the axons (Fig.20H). Consistent with the cell culture data Robo3 and Robo31064-1342 were targeted only to the axonal compartment of MB neurons (Fig.20 F-G).

Next, I asked what if the rate of endocytosis was more than exocytosis of newly synthesized receptor. To test this, I used a temperature sensitive, Shi^{ts}dynamin mutant that blocks endocytosis at temperatures 25 degree and higher (Kosaka and Ikeda, 1983a)(Fig.21). Robo3 and CD8-Robo31064-1342 exhibits similar localization patterns in wild type versus mutant background at higher temperature indicating that endocytosis had no role in Robo3 axon targeting. On the other hand, blocking

dynamin-dependant endocytosis interestingly promoted Robo3 1-1063 axonal localization (Fig.22)suggesting that loss of Robo3 1-1063 localization was due to lack of stability, such that the receptor gets endocytosed after reaching the axonal membrane. Note, the percentage of neurons with CD8-Robo31064-1342 in the axon after 6hrs at 29 degrees is significantly less as compared to full length Robo3. This could be because Robo3 targeting ability is either enhanced in the presence of extracellular domain. I then quantified the receptor expression on the membrane by surface labeling and found the percentage of neurons with Robo3 1-1063expressionwas appreciably more in dynamin mutant background(Fig.23). This indicated that Robo3 1-1063 domain of Robo3 is endocytosed after it reaches the membrane but Robo3 and CD8-Robo3 1064-1342 are rather stable on the membrane.

To examine the role of cytoskeletal tethering in providing stability to the receptor, I performed detergent extraction assay and FRAP experiments. Cytoskeletal associated proteins are known to be non-ionic detergent insoluble at room temperatures (Winckler et al., 1999; Zhang et al., 2012). Ankyrin1 GFP at the barrier,Robo3 (Fig.25) and CD8-Robo3 1064-1342 (Fig.26) in the axon were resistant to detergent Triton X-100 treatment. However, membrane marker proteins and Robo3 1-1063 were successfully extracted with 10min of Triton X-100 treatment at room temperature. This indicated that ankyrin, Robo3 and Robo3 1064-1342 proteins most likely tethered to the actin cytoskeleton butRobo3 1-1063 is not. Phalloidin staining after detergent extraction also stained the F-actin skeleton appreciably (Fig.26F).

Cytoskeletal tethered proteins also exhibit slow planar mobility and thus low florescence recovery rates after photo bleaching due to the presence of large fractions of immobile molecules in the population (Saxton, 1997; Saxton and Jacobson, 1997; Woda et al., 1981)(Fig.27). Thus, florescence recovery rates after photo bleaching (FRAP) were examined for different constructs (Lippincott-Schwartz et al., 1999; Siggia et al., 2000). CD8-GFP (used as control) exhibited a high florescence recovery pattern (80-90%) after bleaching a 1um^2 area in 60 seconds (Fig.28A). However, CD8-Robo31064-1342 GFP (Fig.28B) exhibited reduced recovery (~30% of the total) pattern. Similarly, Robo3 and Robo3 with ITENK mutation were also immobile with florescence recovery of less than half the initial (Fig.30). But, Robo3 1-1063 was found more mobile than Robo3 GFP possibly due to of lack of cytoskeletal tethering (Fig.29). Above results suggest two points; 1. Robo31064-1342 domainis largely involved in receptor tethering that is missing in Robo3 1-1063 and 2. ITENK in the C-terminal cytoplasmic region is not involved in tethering and may be required for axon targeting. Treating Robo3-GFP expressing cells with an F-actin disrupting agent, LatA 10uM for 1hour, notably reduced the fraction of immobile molecules in the population (Fig.31). Reduced mobility of Robo3 receptor on the membrane could be attributed to its localization pattern at the sub compartment level. In contrast to Robo3 1-1063, Robo3 and CD8-Robo31064-1342 with Robo3 C-terminal cytoplasmic domain are found in

characteristic pre-synaptic bouton-like protrusions. In vertebrate neurons such protrusions are known to have enriched levels of F-actin and serve as sites of tethering signalling molecules, synaptic constituents and scaffolding proteins (Shoop et al., 2000; Takahashi et al., 2003; Zhang and Benson, 2001)(Fig.24). Time course analysis of newly synthesized CD8-Robo31064-1342 with ITENK mutation in mature neurons showed that axonal targetingof C-terminal domain requires ITENK. I thus, propose that axonal compartmentalization of Robo3 receptor (and Robo2 based on sequence similarity) is established by polarized targeting and maintained by actin cytoskeletal associations in addition to the ankyrin-based diffusion barrier between axon-dendrite compartments.

ショウジョウバエ神経突起の細胞膜は、近位部と遠位部の２つに区画化されることが知られている。各区画には異なる膜蛋白質が分布し、区画の境界にある拡散障壁がお互いの膜蛋白質の混じり合いを防いでいる。Robo2 と Robo3 は１回膜貫通型の軸索ガイド分子受容体で、ショウジョウバエ培養神経細胞において神経突起の遠位区画に局在する。Joshi さんは、これらの蛋白質の遠位部特異的な局在化機構を解析した。

まず、Joshi さんは、Robo2 と Robo3 の分子解剖を行い、神経突起遠位部への局在に関わる２つのドメインを同定した。一つはＣ末の細胞内ドメインで、このドメインを付加することで、通常は遠位にも近位にも分布する膜蛋白質を、遠位部特異的に局在化できる。このＣ末ドメインの遠位部局在化活性には、Robo2 と Robo3 とで共通する５アミノ酸配列 ITENK が必要不可欠であった。もう一つは細胞外の免疫グロブリン様ドメインで、これ単独では局在化を誘導できないが、Robo 蛋白質の他のドメインと協調することで遠位部局在化活性を発揮する。

Joshi さんは、膜蛋白質の区画選択的な局在化には、1)新規合成の際の区画選択的な細胞膜への挿入と、２）細胞膜挿入後の細胞骨格による安定化 の２つの機構が関与すると考えた。そして Robo 蛋白質の局在化ドメインが、これらの機構において機能するかについて検討した。その結果、Ｃ末の細胞内ドメインが、1) 区画選択的な細胞膜への挿入　2)細胞骨格による安定化 の両方で機能することがわかった。このドメイン中のアミノ酸配列 ITENK は、遠位部選択的な細胞膜への挿入に必須であり、挿入先を決定する重要な新規モチーフである事が示唆された。一方の細胞骨格による安定化については、ITENK 以外のＣ末の細胞内ドメインが働く事がわかった。

以上の結果に加えて、Joshi さんは、区画の境界部に、細胞膜蛋白と細胞骨格とのアダプター蛋白質である Ankyrin1 が局在する事を見いだしている。Ankyrin は、哺乳類の神経細胞において軸索起始部の拡散障壁を形成すると考えられており、このアダプター蛋白質がショウジョウバエ神経突起においても拡散障壁の原因となる可能性が示唆される。また、Joshi さんは、前シナプスマーカーがショウジョウバエ培養神経突起遠位区画に局在する事も見いだしており、哺乳類神経細胞とのアナロジーから、ショウジョウバエ神経突起の近位部と遠位部はそれぞれ樹状突起と軸索に相当すると提唱している。

以上の研究結果は、一続きであるはずの神経突起細胞膜が区画化される過程に、複数の機構が関わる事を明らかにしたものであり、その分子機構について数多くの新規知見を提供した。したがって博士号授与の要件を満たすと審査員全員一致で判断した.