Review

Molecular basis of the functions of the mammalian neuronal growth cone revealed using new methods

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Abstract: The neuronal growth cone is a highly motile, specialized structure for extending neuronal processes. This structure is essential for nerve growth, axon pathfinding, and accurate synaptogenesis. Growth cones are important not only during development but also for plasticity-dependent synaptogenesis and neuronal circuit rearrangement following neural injury in the mature brain. However, the molecular details of mammalian growth cone function are poorly understood. This review examines molecular findings on the function of the growth cone as a result of the introduction of novel methods such superresolution microscopy and (phospho)proteomics. These results increase the scope of our understating of the molecular mechanisms of growth cone behavior in the mammalian brain.

Keywords: axon guidance, proteomics, phosphorylation, superresolution microscopy, lipid rafts, chondroitin sulfate

1. Introduction

The neuronal growth cone is a highly motile structure that is specifically formed at the tips of developing axons and dendrites. This structure was discovered and first described by the distinguished anatomist Santiago Ramón y Cajal, who was the first neuroscientist, along with Camillo Golgi, to win a Nobel prize.[1] This review mainly focuses on the axonal growth cone, because the dendritic growth cone is not well characterized.

A pioneering study using cell culture revealed that the growth cone actually moves when the neurite is growing,[2] and a live-imaging study (movie) by Junnosuke Nakai revealed that growth cone movement is regulated by multiple cues.[3] Molecular and cellular biology studies have shown that F-actin initiates growth cone motility,[4] and genetic approaches to neural wiring[5,6] have contributed to the molecular understanding of growth cone behav-

ior. Axon guidance molecules, such as netrin, semaphorins, and ephrins, are localized outside the growth cone, and signaling in the growth cone occurs in response to such molecules. In addition, each guidance molecule frequently induces opposite movements in the direction of the growth cone, *i.e.*, attraction or repulsion. Thus, determination of growth cone direction is dependent upon the signaling in it rather than the guidance molecules themselves.

The fundamental mechanisms of other neural development processes, such as regional determination, neural cell lineage, neurogenesis, neuronal migration, and synaptogenesis in the mammalian central nervous system (CNS), are relatively well understood due to investigations over the last quarter century. However, the fundamental molecular machinery involved in growth cone functions in the brain of higher organisms remains poorly understood.

Most growth cone studies have been primarily performed using (1) model organisms, such as *Caenorhabditis elegans* or *Drosophila*; (2) other invertebrate neurons, such as those from *Aplysia*; or (3) peripheral nerves of vertebrates, such as chick dorsal root ganglion (DRG) neurons. These studies have contributed to progress in this field when molecular and cellular neurobiological techniques

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using mammalian CNS neurons are not sufficiently feasible. However, the results derived from model organisms or the chick DRG cannot be simply applied to the understanding of molecular pathways in the mammalian CNS growth cone, because mammalian signaling mechanisms are generally much more complex and have greater diversity than those of simpler neuronal systems. To approach this problem, we utilized methods that are readily available for directly determining the numerous protein components in the growth cone of the mammalian CNS.

Currently, mammalian CNS growth cones are the most intensively studied. Clearly, the growth cones of simpler organisms or those outside the CNS (such as DRG) have completely different molecular compositions and interactions compared with the mammalian CNS. Simple hypotheses have been proposed from studies using non-mammalian growth cones. These hypotheses, especially the molecular aspects, should be tested to determine whether they apply to the mammalian CNS. Over the last decade, mammalian CNS neurons, such as cortical or hippocampal neurons, have been utilized to discover additional molecular signaling pathways. Because hippocampal and DRG neurons differ considerably from each other,[7] the molecular mechanisms of their growth cone behavior are also likely to be considerably different.

Recently, new techniques have been introduced in studies on the molecular mechanisms of mammalian growth cone behavior, and this review summarizes the results of the new findings.

2. Basic structure and signaling of the growth cone

In the growth cone, microtubules (MTs) and various vesicles are concentrated near distal axonal shafts, a region called the central (C-) domain of the growth cone.[8] These vesicles function as plasmalemmal precursors for axonal growth, although the details of their turnover and recycling are not well understood.[9] In addition, some vesicles may be endosomes derived from endocytosis.[10] Although morphological vesicular categorization in growth cones was reported previously using electron microscopy, molecular markers that discriminate among these have not yet been determined.[8),11),12] Vesicular trafficking in growth cones is important not only for maintenance of the plasma membrane but also for axonal guidance.[11),13)–15]

The area near the leading edge of the growth cone, called the peripheral (P-) domain,[8] has a high concentration of dynamic F-actin, and only this domain enables the growth cone to move. The coordination between F-actin rearrangement in the P-domain and MT growth in the C-domain is essential for growth cone function, but the molecules that link these two have not been defined exactly.[16),17] In the P-domain, branched F-actin forms lamellipodia, whereas unbranched filaments form slender protrusions, called filopodia. In *Aplysia* growth cones, which are much larger than mammalian ones, a transition zone (T-zone) is clearly discriminated between the C- and P-domains.[18),19] However, this zone is not distinctly recognized in mammalian growth cones. This review will not discuss the T-zone further.

In the C-domain, the cortical membrane skeleton, which is composed of F-actin, spectrin, and other components (discussed below[13]), is enriched underneath the plasma membrane. These structures are likely important for changes in the structure of the growth cone when the axon grows and the new MTs invade the growth cone area. However, F-actin dynamics and the molecular mechanisms involved in this process have not yet been analyzed.

The distribution of organelles in the growth cone has not been described clearly, even using electron microscopy. Growth cones contain mitochondria, a few clusters of ribosomes, branched membranous reticulum, lysosomes, cytoskeleton,[16] membranous disks, and vacuoles.[17] Some of these structures have been demonstrated by identification of molecular markers using proteomics.[13),20] Local protein synthesis by the ribosomes in growth cones has been demonstrated and may be involved in determination of their advancing direction[21] (see below section **3.4**).

Two second messengers are present in the growth cone, Ca^{2+} and $cAMP$[12),22)–24] (see also a review;[25] Fig. 1). These second messengers are involved in signaling induced by axon guidance molecules such as netrin, semaphorins, or ephrins.[12),25] Diffusible factors are also attached to the cell surface/extracellular matrix to guide the growth cone. Thus, *in vivo* signaling including local translation mechanisms should be revisited, and the classical gradient theory should be revised.[26),27]

3. Proteomics profiling of the mammalian growth cone

As mentioned above (see **1. Introduction**), complete molecular profiling of the mammalian CNS growth cone is needed for understanding of
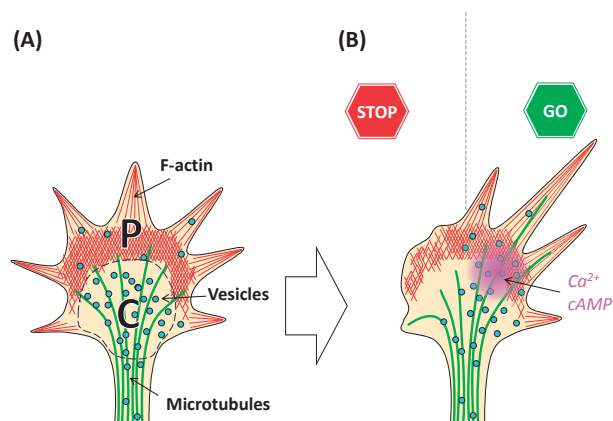
(A) **(B)**

Fig. 1. The growth cone structure (A) and its behaviors are regulated by second messengers (B). The growth cone is divided into two regions; one is the central (C-) domain in which MTs and vesicles are enriched; the other is the peripheral (P-) domain in which dynamic F-actin bundles are enriched, forming lamellipodia and filopodia. Growth cone behavior is regulated by axon guidance molecules via up- and down-regulation of second messengers such as Ca^{2+} and cAMP. Axon guidance molecules are either attractive ("GO") or repulsive ("STOP") to the growth cone, probably via higher and lower concentrations of second messengers for attractive and repulsive directions, respectively.

the molecular basis of growth cone function. Proteomics analysis, which quantitatively identifies numerous proteins, has completely overcome previous methodological limitations for determining protein components.[28] We performed a proteomics study of mammalian growth cones collected by subcellular fractionation.[29]–[32] Following subcellular fractionation, isolated growth cones or growth cone particles (GCPs) were enriched, and hypotonic treatment of this fraction provides growth cone membranes (GCMs).[31] Using these methods, we characterized many previously unknown growth cone proteins.[20] After our report, another group identified additional GCP proteins by proteomics,[33] and almost all of our results were validated in their report. Surprisingly, although we performed immunostaining for more than 200 proteins, no false positive cases were found. These results confirmed the extremely high purity of our GCP/GCM fractions.[20]

In contrast to synapse-associated proteins in mature synapses,[34]–[36] molecular markers for growth cones, except for the classical neuronal growth-associated protein, GAP-43, have not been established.[37] We identified approximately 20 proteins that are functional molecular markers of the growth cone; these were termed neuronal growth-associated proteins (nGAPs) and are involved in the functions

of mammalian CNS growth cones.[20] Initially, we were slightly surprised that few proteins were found compared with the number involved in neurite growth in *C. elegans* or *Drosophila*. The reason for this difference was that these proteins in lower organisms are not generally concentrated in their nervous systems in contrast to the mammalian CNS. This finding suggested that the molecular mechanisms of mammalian neuronal growth differ considerably from those of simple organisms.

Because all putative nGAPs from cortical neurons satisfied the criteria of "nGAPs", even in PC12D cells,[38] nGAPs (Fig. 2) are considered universal growth cone markers that are not dependent on neuronal species. Recently, some proteomics studies of developing neurons have been published.[39],[40] They included information about locally synthesized proteins. These data will be useful for understanding the molecular aspects of growth cone functions. In addition, the proteomics techniques employed by another group also were also useful for identification of a specific new protein called shootin-1, which regulates F-actin during axon growth.[41] Characteristics of the growth cone proteins identified by proteomics have been described in detail previously.[13] Thus, only recently identified proteins are summarized here.

3.1. MTs and MT-related proteins. Two types of MT-binding proteins are present in the growth cone. Microtubule-associated proteins (MAPs) are localized adjacent to MTs and stabilize them. MAP1B and tau are mainly localized in the mammalian growth cone.[20] MAP6, also called STOP protein, was identified as a MAP with growth cone proteomics,[20] and this protein is involved in rapid axon growth for neuronal polarity determination through palmitoylation[42] (discussed below). However, curiously, this protein has functions that are independent of MT binding. Through its binding to SH3 domain-containing proteins, MAP6 also acts as a downstream signaling protein of semaphorin-3E.[43]

Plus-end interacting proteins (+TIPs) of MTs are a completely different protein group that selectively bind to MT (+)-ends at selected polymerization sites distinct from MAP-binding sites. +TIPs are composed of many subgroups, each of which is structurally different.[44] These proteins are considered strong candidates for proteins that link MTs and F-actin in the growth cone.[45] TACC3 is a minor +TIP that promotes axon elongation.[46]

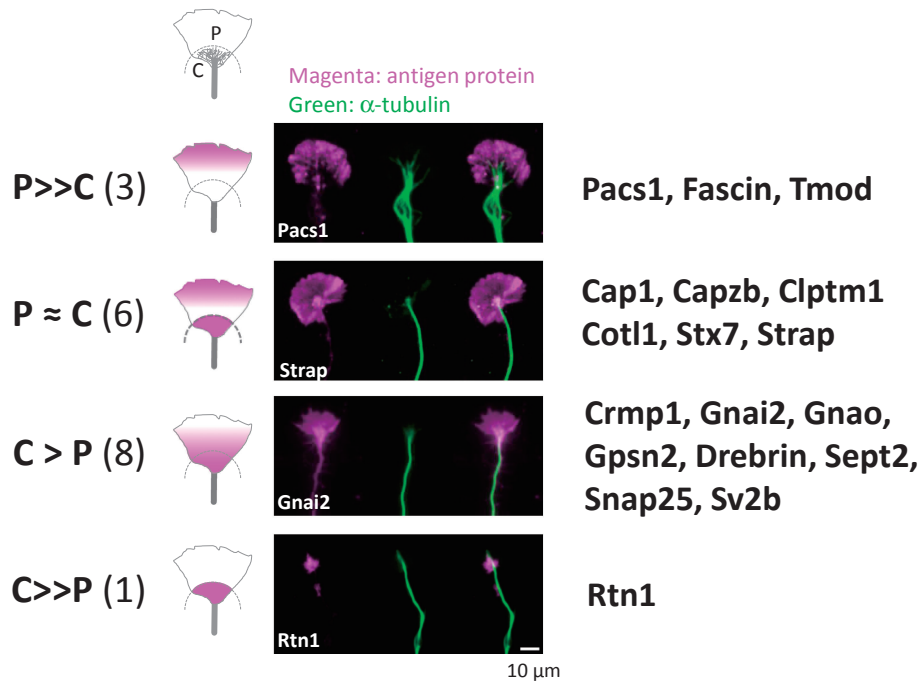**3.2. Actin filaments and actin-binding proteins.** As described previously, filopodia and

Fig. 2.   The nGAPs identified in our proteomics studies.[20] C: central domain; P: peripheral domain. P ≫ C, P-domain-dominant localization; P ≈ C; evenly distributed both in C- and P-domains; C > P, enriched in the C-domain compared to the P-domain; C ≫ P, C-domain-dominant localization.

lamellipodia, in which the dynamic actin filaments are concentrated in the P-domain, are present in the growth cone. The actin polymerization and depolymerization cycle is believed to determine the direction of axon growth.[47] By proteomics analysis of the growth cone, we characterized the actin-binding proteins.[13),20] The classical idea of growth cone motility based upon filopodial F-actin is summarized in several reviews.[48] Three types of actin isoforms, α-, β-, and γ-actin, are localized in the growth cone.[20] α- and β-actin are involved in axonal filopodia dynamics and collateral branch formation, and dynamic movements of growth cone filopodia, respectively.[49] The shapes of the growth cone are generally regulated by F-actin organization.[50)–53]

The single-headed myosin, Myo1b, which is present in the growth cone,[13),20] is a necessary component for axon growth via actin waves from the cell body.[54] Reports disagree regarding whether actin waves are physiologically involved in neuronal development or not;[55),56] thus, further studies are needed to investigate this idea.

3.3. Signaling proteins.
3.3.1. Protein kinases.   Our proteomics data[15] showed that protein kinase A (PKA) and Ca^{2+}/

calmodulin-dependent protein IIβ (CaMKIIβ) are the major protein kinases in the growth cone. Thus, as mentioned above, cAMP and Ca^{2+} are the postulated second messengers in the growth cone. However, how these second messengers are increased and what types of biochemical mechanisms are involved are not yet clear. For example, the levels of voltage-gated Ca^{2+} channels are much lower than in mature synapses, and most Ca^{2+} channels in the growth cone are L-type channels.[20] A recent report using a new Ca^{2+} indicator supported the idea that this type of Ca^{2+} channel is important for nerve growth.[57]

3.3.2. Heterotrimeric G proteins (G proteins) and G-protein coupled receptors (GPCRs).   Heterotrimeric G-proteins are highly concentrated in the growth cone.[13),20),58)–60] However, the significance of G-proteins has not been characterized well due to the low abundance of GPCRs.[13),61] The human autosomal recessive neurological disorder Chudley–McCullough syndrome is caused by defective Gpsm2/Gαi3 signaling in stereocilia, which causes disruption of actin dynamics in the growth cone.[62] The precise signaling pathways upstream and downstream of GPCRs should be analyzed regarding growth cone functions. In addition, the GPCR

latrophilin-1 is involved in short-range axon pathfinding by interacting with the proteolytic fragment of the membrane protein Lasso/tenurin-2.[63] Latrophilin-1 is a presynaptic adhesion GPCR protein in the mature neuron,[64] but this protein is also present in the growth cone.[13),20] How the G-proteins are related to this process may be important for local axon attraction/repulsion.

3.3.3. Rab family. Many Rab proteins are present in growth cones,[20] and some reports suggest that they are involved in nerve growth.[65),66] However, their direct functions or interactions regarding axon growth have not been clarified.[67] In contrast to other small GTPases (Ras/Rho family proteins) in the growth cone, the activation and inactivation mechanisms of these Rabs, such as their activators (guanine nucleotide exchange factors) or inactivators (GTPase-activating proteins), are not known. Rab11, a recycling marker, supports axon regeneration;[68] thus, how Rab proteins work in the growth cone should be studied further.

3.3.4. Adapter molecules.

(a) CRMP family. CRMP2 was first characterized as a neuronal polarity-dependent protein,[69] and CRMP4 is involved in cytoskeletal rearrangement for axon growth/regeneration.[70),71] The CRMP family includes the most important and abundant adapter molecules. In addition, CRMP2 interacts with Sra-1/CYFIP, which is a Rac-associated regulator of the cytoskeleton, and regulates axon formation.[72] Because CYFIP is an nGAP,[20] this report is quite interesting.

(b) 14-3-3 family members. The 14-3-3 family includes adapter proteins for proteins phosphorylated by serine (S)/threonine (T) kinases, in particular, PKA.[73] Among the many members of this family, we confirmed that β, ε, ζ, η, γ, and θ forms are abundantly present in GCPs. 14-3-3-dependent signaling is thought to be a major event downstream of PKA in the growth cone,[74] and a chemical compound fusicoccin-A is a stimulator of axon regeneration.[75]

3.3.5. Protein translation machinery (Fig. 3). The growth cone has the potential for local protein synthesis, including initiation factors, elongation factors, and ribosomal components.[13),20] Local protein synthesis there is believed to modify local axon guidance,[12] but not axonal growth itself.[76] This hypothesis is supported by the observation of anterograde axonal transport derived from massive protein synthesis in the cell body. These large amounts of proteins are probably directly utilized for axon growth. In *Xenopus*, many locally synthesized proteins in developing axons have been identified.[76]

3.3.6. Protein degradation systems. Most TRIM family members are known as E3 ubiquitin ligases. TRIM9 is an E3 ligase that is involved in filopodial actin reorganization.[77] TRIM46 lacks ubiquitin ligase activity, but this protein interacts with MTs and controls MT reorganization.[78]

3.3.7. Proteins involved in exocytosis. Our group and others have shown that nerve growth is mediated by the SNARE proteins and related proteins.[79)–82] These proteins are most likely involved in vesicular fusion and targeting in the growth cone.[9]

Syntaxin-1 is involved in axon guidance through guidance molecules[83)–86] and is conserved in a wide range of organisms.[87] Many types of syntaxin-binding proteins have been characterized,[35] including its most abundant partner, Munc-18-1, in the growth cone.[20] Other partners for axon growth are not well characterized.

4. Phosphoproteomics reveals new signaling pathways in the growth cone

4.1. General profiling of GCM phosphoproteomics. To further investigate molecular signaling in growth cones, this review will now focus on protein phosphorylation, the most important regulatory mechanism in many cellular processes[88] and in physiological and pathological conditions in neuroscience.[89] Many reports about phosphorylation-dependent pathways in the growth cone have been published.[90] However, most studies have used *in vitro* phosphorylation systems that do not necessarily represent the *in vivo* situation. In addition, ∼500 protein kinases are encoded in mammalian genomes, and determination of the specific activating kinase is difficult.[91] Currently, phosphoproteomics is an important and powerful method for comprehensive and quantitative identification of *in vivo* phosphorylation sites.[92)–94]

We performed phosphoproteomics analyses of GCMs[31] and identified more than 30,000 phosphopeptides and ∼4,600 different phosphorylation sites from ∼1,200 proteins.[95] These phosphorylation sites are likely those that are necessary for *in vivo* growth cone activity. Surprisingly, most of the highly frequent phosphorylation sites (>100 times) were previously uncharacterized.[95] These results indicate that a large number of unknown, potentially important phosphorylated sites are involved in mammalian growth cone functions.
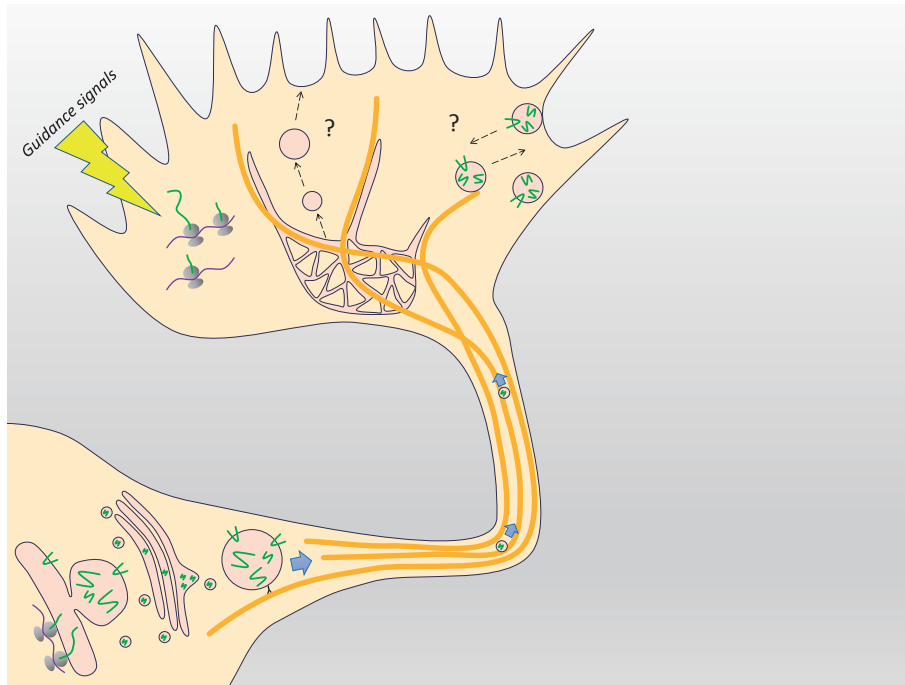
Fig. 3. Protein usage in the growth cone. Proteins used in the growth cone for axon growth are mainly transported axonally after their synthesis in the cell body. However, some proteins are locally synthesized in the growth cone in response to axon guidance signals. Transported vesicular proteins probably undergo recycling, although most of the precise mechanisms and dynamics of these processes are uncharacterized.

S or T protein kinases are categorized into four classes: acidic, basic, proline (P)-directed, and other kinases, based on the neighboring amino acid residues of the phosphorylated S or T.[96] We showed that more than 60% of S or T phosphorylation sites in GCMs were P-directed phosphorylation. We applied bioinformatics analyses to these data and revealed that these frequently activated P-directed kinases were mitogen-activated protein kinases (MAPKs).[95] The MAPK family is composed of three subfamilies: extracellular signal-regulated kinase, c-Jun N-terminal kinase (JNK), and p38. Inhibitor studies determined that JNK is the most likely kinase responsible for the highly frequent phosphorylation sites.[95] This was unexpected because JNK is an apoptotic signal in many cells,[97] and in neurons, JNK induces axon degeneration.[98]

We have reported that at least seven highly phosphorylated sites in MAP1B (two sites), GAP-43 (two sites), SCG10,[99] Rufy3,[100),101] and Robo2 are JNK dependent.[95] These results indicated that JNK activation is physiologically required in the mammalian growth cone via phosphorylation of proteins that belong to the "actual working unit" for axon growth (Fig. 4).

JNK phosphorylates many kinds of proteins that are directly involved in axon growth/regeneration.[95] A decade ago, reports described that normal brain development requires the activation of JNK,[102)–106] and adult neuronal plasticity also involves JNK activity.[107)–109] JNK plays a role in retrograde axonal transport and phosphorylates transcription factors in the nucleus.[110] However, our results on phosphoproteomics revealed that JNK phosphorylates many proteins involved in axon formation.

Several lines of evidence suggest that the developing neuron has high JNK activity, which is essential for the physiological development of the mammalian brain.[106),111] In addition, axon guidance and regeneration, which are dependent on netrin-1, likely also require JNK activation.[112),113] Why the high enzymatic activity of JNK does not induce apoptosis is not clear. Our phosphoproteomics results provided an unbiased demonstration that nerve growth during development in the mammalian brain requires JNK activity and involves a large number of its substrates, which belong to various categories.

4.2. Phosphorylation of GAP-43. In our GCM phosphoproteomics study, the most abundant phosphorylated site was S96 of the classical growth
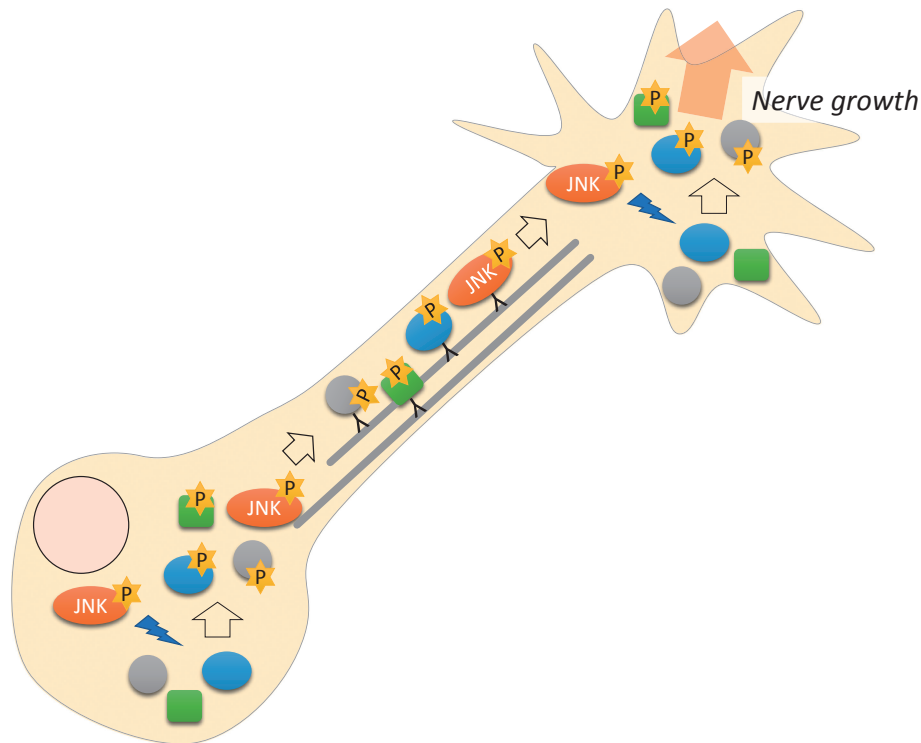
Fig. 4. JNK probably phosphorylates various proteins that are directly necessary for neuronal growth. JNK is activated in the cell bodies, but active JNK may undergo anterograde axonal transport. Phosphorylated proteins are also thought to be transported from the cell body to the growth cone, although some may be phosphorylated locally in the growth cone.

cone marker GAP-43,[95),114),115)] comprising more than 1% of all phosphopeptides. This phosphorylated site had only been reported once, more than a quarter of a century ago,[116)] just before the discovery of JNK itself.[117)] As a result, S96 and JNK were never related to each other until our study.[95)] S96 phosphorylation (pS96) is JNK dependent. A pS96-Ab specifically recognizes growing axons *in vivo* during certain developmental stages.[95)] In addition, pS96 is detected in regenerating axons after sciatic injury, not only by immunohistochemistry,[95),118)] but also directly by mass spectrometry.[95)]

GAP-43/neuromodulin was one of the most featured proteins in vertebrate axon growth/regeneration before its knockout (KO) was produced.[119)] Contrary to the expectation from cellular findings, no large changes in nerve growth or neuronal connections were found in the KO line. The phenotype of these mice reduced researchers' interest in this protein, and this protein has received little attention using the new technologies in neuroscience.

GAP-43/neuromodulin has several unique biochemical properties. (1) This protein is an a intrinsically disordered protein. The protein does not have specific high-order structures, but acquires specific functions or interactions after modifications such as phosphorylation. A recent nuclear magnetic resonance study[120)] confirmed this idea for GAP-43. (2) This protein is palmitoylated (C3C4) and attached to membranes, probably to the plasma membrane.[121)] (3) GAP-43/neuromodulin is the first characterized calmodulin-binding protein, via its IQ domain/motif.[115)]

Protein kinase C-dependent S41 phosphorylation of GAP-43, a previous area of study, has not been identified in the growth cone.[95)] This site is not detected in regenerating axons; thus, we concluded that its phosphorylation does not direct axon growth/regeneration, although a number of studies referred to these relationships more than 20 years ago.

Unsolved: Why are a large number of growth cone proteins, which should support nerve growth, simultaneously phosphorylated by JNK? This is the most important problem to solve.

5. Superresolution microscopy reveals associations between F-actin organization and membrane trafficking in growth cones

To better understand the molecular basis of neuronal growth, the precise relationship between both cytoskeletal and membrane components must be clarified. In addition, the mammalian CNS growth cone is three-dimensional, compared with those of the peripheral nervous system or invertebrate neurons, the growth cones of which are flat. However, little progress has been made despite the 3D elucidation of the growth cone structure. Unfortunately, the diffraction limitation of conventional optical microscopy (∼200 nm), including conventional confocal microscopy, precludes such analyses.[122),123)] Several types of superresolution microscopy (structured illumination microscopy [SIM], stimulated emission depletion microscopy [STED], photoactivation localization microscopy [PALM], stochastic optical reconstruction microscopy [STORM], *etc.*), which are based upon different principles, have succeeded in overcoming the optical diffraction limit and have achieved a resolution of 50–100 nm.[123)] Superresolu-

tion microscopy has revealed a newly recognized distribution of F-actin in the axon, which is implicated in axonal transport, polymerization of F-actin,[124)–127)] and the distribution of tau and MTs in growth cones.[128)]

SIM can be used to visualize the fine structure of cells by calculating the interference (moiré) patterns induced by irradiation with striped-pattern excitation light.[129)] Using SIM, approximately 100 nm in lateral dimensions and 300 nm in the axial dimension can be visualized. Superresolution images are easy to obtain with SIM, because it utilizes typical fluorescent dyes.[130),131)]

Using SIM, we determined the precise localization of a number of proteins identified by growth cone proteomics.[20),132)] Our analysis using SIM revealed a new linkage between the organization of F-actin bundles and local endocytosis at the leading edge,[132),133)] which could never have been acquired using confocal microscopy. Immediately after publication of the proteomics results, we began to analyze vesicular movements for live imaging. However, the dense distributions of growth cone vesicles prevented the measurement of quantitative data using confocal microscopy (Fig. 5).
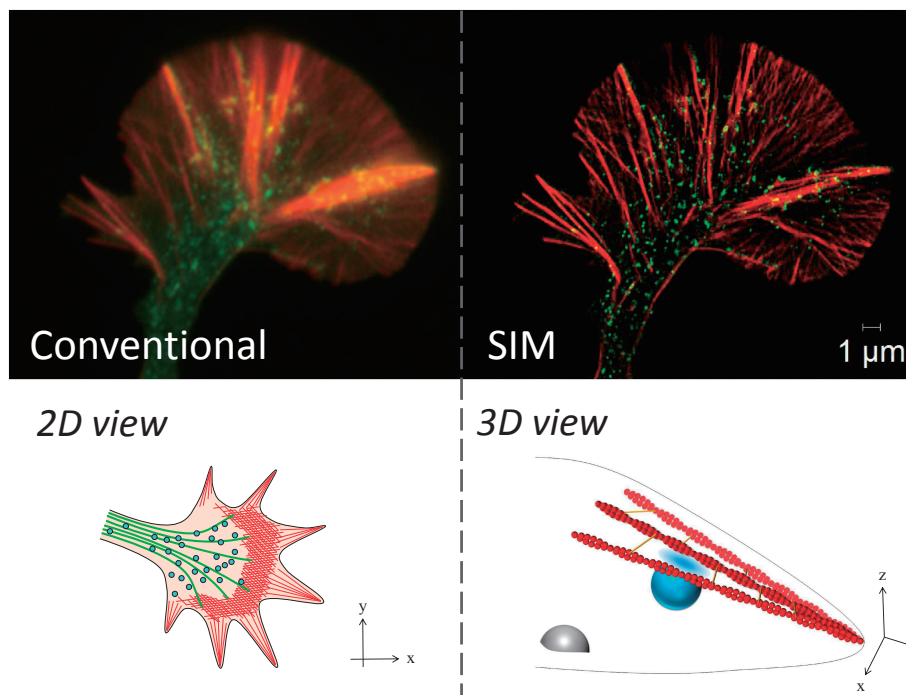

Fig. 5.   Superresolution microscopy reveals new endocytotic sites for nerve growth. *Red*, mCherry-actin; *Green*, EGFP-synaptophysin. Conventional confocal microscopy view (*upper left*) is not suitable for visualizing the densely packed cytoskeleton and vesicles. In addition, confocal microscopy is generally used to acquire two-dimensional views (*lower left*). SIM superresolution can be used for clear, 3D acquisition of the distribution of the growth cone cytoskeleton and vesicles (*upper right*).

These results have important implications. First, vesicular movements in the P-domain were clarified. From classical studies, vesicles in the C-domain were thought to be fused and undergo recycling.[8),15)] However, vesicles in the P-domain do not fuse with each other, and they move toward the C-domain.[8)] These previous studies suggested that vesicles in the C- and P-domains have different dynamics and that their molecular characteristics may be different. Here, we clarified vesicular movement in the P-domain directly and determined the significance. Second, we studied coordinated movements of the vesicles and the actin cytoskeleton for axonal growth in the growth cone. We also clarified the involvement of key molecules in this process, *i.e.*, dynamin, endophilin, and fascin. Fascin is an actin-binding protein that forms F-actin bundles and is one of the nGAPs we identified.[20)] Third, the newly identified type of endocytosis in the P-domain, which requires dynamin and a BAR domain-containing protein (which regulates membrane curvature), endophilin, is similar to fast endocytosis[134)] and occurs in the z-axis direction. Finally, some of the F-actin in the P-domain is also distributed three-dimensionally.[122),132)]

Unsolved: The first problem is where vesicular fusion occurs for plasma membrane expansion in axon growth. If can we observe movement of the growth cone in the z-axis direction, we may find the answer. Second, the significance of recycling for axonal growth is not yet known.

6. Lipid rafts in the growth cone and neuronal polarity signals

In various cells, lipid-raft domains, in which sphingolipids and cholesterol are highly concentrated, are believed to concentrate signaling molecules in the plasma membrane as a result of their lower fluidity than in other areas.[135)] Thus, lipid rafts are currently thought to be the "hot spots" for signal transduction in cells other than neurons. However, because the neuron has a much higher concentration of these lipid species than other cells,[136)] lipid rafts are likely to be important in neuronal signaling. Some membrane proteins are highly palmitoylated,[137)] which causes partitioning of membrane lipids and forms lipid rafts, incorporating such proteins.[138)–140)] A human mutation in serine palmitoyltransferase, the enzyme that catalyzes the first step of sphingolipid synthesis, causes abnormal growth cone dynamics, implicating the importance of this enzyme in neuronal development[141)] (Fig. 6a).

Our group has focused on the unique membrane protein glycoprotein, glycoprotein m6a (GPM6a), and found that lipid rafts are important for axonal growth.[100),101),142)] Our proteomics study indicated that GPM6a is a major membrane protein of GCM.[20)] GPM6a is highly expressed in differentiated neurons,[143)–145)] although its exact function is not known. This protein has four transmembrane domains[146)] and is a major palmitoylated protein in the adult brain.[137)] We found that GPM6a is highly colocalized with cholesterol, a marker of lipid rafts.[100)] Additionally, lipid rafts containing GPM6a are clustered, and GPM6a itself accumulates at the origin of the neurite.

The location of growth cone formation at a specific portion of a neuron is an important unsolved problem in neuronal development. The steps in which the neuron extends processes, *i.e.*, the axon and dendrites, during differentiation are classified into five stages in cell culture. Such an event is called neuronal polarity determination (Fig. 6b). Although the characterized cell-autonomous signaling pathways may determine neuronal polarity in individual single cells in a cell culture model system,[147)] as postulated by Banker and colleagues,[148)] according to the tug-of-war model,[149)] such signaling does not likely occur spontaneously and in an inconsistent way during polarization of neurons *in vivo*.[150)] *In vivo*, the neurons in one group should acquire their polarity simultaneously and grow their axons in the same direction. Thus, we need a new scheme that explains how extrinsic factors induce polarity determination. Two factors are known; one is the neurotrophin group,[147)] and the other is the extracellular matrix (ECM) component group, which includes laminin (LN).[100),151)] We revealed that LN facilitates neuronal polarity determination, which appears to skip stage 2.[100)] Such LN-induced polarity is dependent upon palmitoylated GPM6a, which is concentrated in lipid rafts. Without palmitoylation, GPM6a is localized in the plasma membrane but not in lipid rafts. As a result, neither its biased distribution at the initial growing site nor facilitation of polarity determination is induced.[100)]

As mentioned above, we demonstrated that palmitoylated GPM6a clusters in lipid rafts and its biased distribution is consistent with the origin site of the first neurite, in response to LN.[100),101)] Its downstream signaling proteins, Rufy3 (also called Singar1[152)]), Rap2, and Tiam2/STEF, each of which is involved in cellular polarization, are concentrated in lipid rafts in a GPM6a-dependent manner. *In utero*
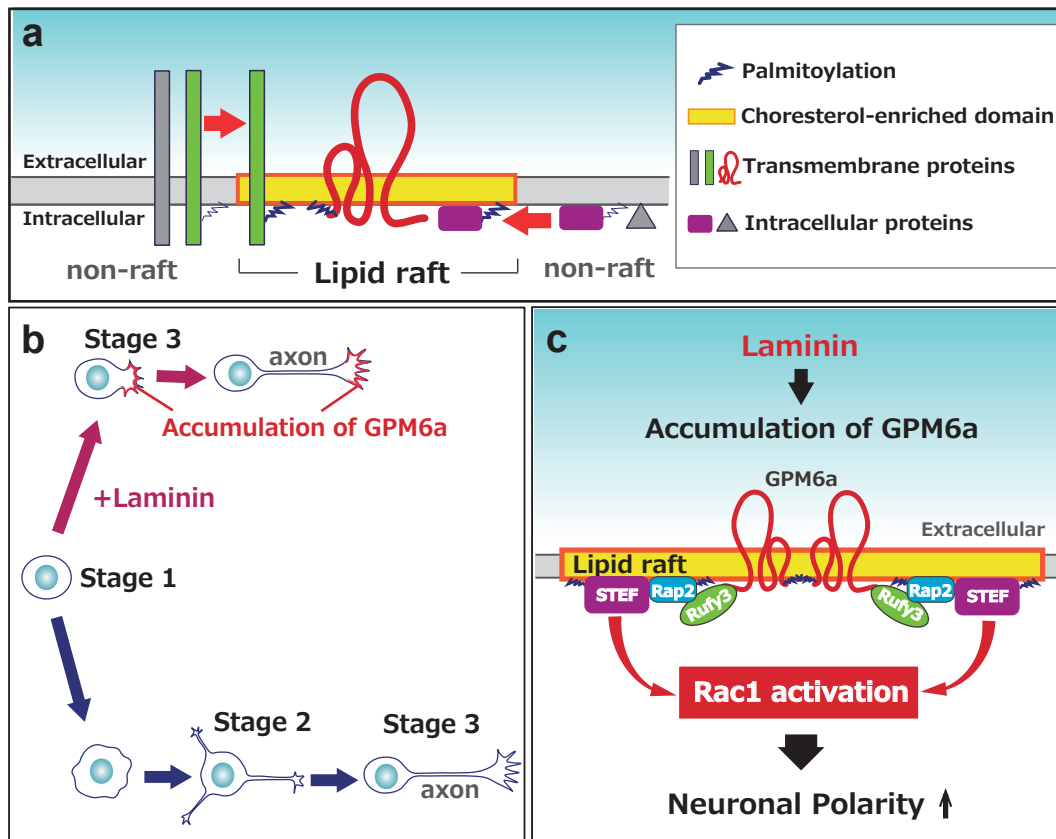
Fig. 6. Lipid rafts and palmitoylation are likely involved in neuronal development, especially in determining neuronal polarity and axon formation. **(a)** Lipid raft structure. Palmitoylated membrane proteins are translocated to lipid-raft domains from non-raft areas. **(b)** Polarity determination of neurons. Neurons are thought to have the potential for cell-autonomous polarity determination via stage 2 (multi-polar stage). However, LN- and GPM6a-dependent processes facilitate the polarity determination process by skipping stage 2 and directly moving to stage 3 (axon determination). **(c)** GPM6a-dependent signaling for polarity determination. Downstream proteins of GPM6a (Rufy3-Rap2-Tiam2/STEF) are gathered to lipid-raft domains in response to LN to facilitate signaling needed for neuronal polarity determination.

administration of RNAi against GPM6a impairs neuronal polarity in pups.[100] These results suggested that GPM6a induces the clustering of downstream signaling molecules in the rafts for more rapid determination of neuronal polarity and axon formation, in response to extracellular signals[100] (Fig. 6c).

Because the growth cone has lower levels of cholesterol and sphingolipids than adult synaptic membranes[153] and the lipid species that compose the lipid rafts are present in much lower amounts during development than in the mature brain,[154] the enrichment of signaling molecules in lipid rafts is likely to be important for the function of growth cones.

The driving of intrinsic signaling pathways such as GPM6a and its downstream signaling proteins that are concentrated in lipid rafts is likely to be important for rapid and synchronized polarity determination,[147),151),155),156] which may explain why lipid rafts are thought to be important for axonal growth.[157] LN probably stimulates the lipid-raft domains to cluster themselves and to increase these areas via GPM6a,[100] as other guidance molecules seem to do.[158),159]

We also succeeded in visualizing lipid rafts using a labeled cholesterol-binding protein and superresolution microscopy.[132] The most important results, which were unexpected, were that GPM6a rescued its KO neurons in polarity determination, but the mutant-deficient palmitoylation site had no rescue activity, even though they were sufficiently expressed in the membrane.

GPM6a, Rufy3, Rap2, and Tiam1 are related to important neuropsychiatric diseases.[160)–163] In par-

ticular, mutations in genes for GPM6a and Rufy3 are associated with a high risk for human schizophrenia and depression, respectively. These results suggest that GPM6a and downstream molecules play physiological roles in neuronal development. GPM6a in lipid rafts is likely involved in a key step of neuronal morphogenesis.

Unsolved: GPM6a-KO and GPM-6a/6b-double KO mice have fewer abnormalities in the connections *in vivo* than expected from cellular studies.[164] Other membrane proteins with different primary structures from the proteolipid protein (PLP) family, but which are concentrated in lipid rafts, probably compensate for GPM6a loss in the developing brain. The functions of Rufy3 seem to be various and operate via more complicated molecular interactions.[165]–[167] However, these functions are not well understood, because the Rufy3-KO is lethal just after birth without large CNS abnormalities.[101]

7. Axon regeneration via growth cone movement *in vivo* is regulated by chondroitin sulfate

The ECM is tightly involved in axon growth and guidance at developmental stages and successful axon regeneration. The ECM component protein, LN, is a major positive regulator of axon growth via growth cone signaling. In addition to LN, other key regulators of axon growth, including glycosaminoglycans, which are long-repeated disaccharides covalently linked to specific core proteins, are localized in the ECM. Chondroitin sulfate (CS) and heparin sulfate (HS) have related structures, but they play considerably distinct roles in growth cone behavior, *i.e.*, CS and HS have an inhibitory and a stimulatory effect on axon growth, respectively.[168] The physiological effects of CS/HS are not well characterized because good model mice for them are not available. However, some recent investigations have revealed that, for example, HS plays an important role in synaptogenesis via other membrane proteins.[169],[170]

We have also studied CSGalNAcT1-KO (T1KO) mice, which lack the rate-limiting enzyme for CS synthesis.[121],[171] In the adult brain, CS is enriched in perineuronal nets, which surround inhibitory synapses, regulate the activity of GABAergic interneurons (so-called "parvalbumin neurons"), and are involved in GABAergic synaptic plasticity.[172] T1KO mice show microscopic abnormalities in the CNS including perineuronal net insufficiency and several behavioral abnormalities,[173]–[175] but they are fertile and viable[171],[176] with some connective tissue abnormalities.[177]

Because CS is the most abundant and potent inhibitor of axon growth and regeneration,[178],[179] we investigated axon regeneration after spinal cord injury (SCI) in T1KO mice. CS is synthesized by reactive astrocytes,[180] which act bidirectionally on axon regeneration because they block the leakage of macrophages, which clear debris after injury, but are also toxic to the normal remaining axons. Previously, almost all studies investigating the roles of CS in SCI used bacterial chondroitinase ABC, an enzyme that degrades CS.[181] However, CS is necessary in injured neural tissues and, complete loss of CS by chondroitinase ABC does not induce only "beneficial" effects. The inhibitory effects of CS on the growth cone are thought to include several signaling pathways.[177],[182] Axonal regeneration in SCI is inhibited at least via a CS receptor, the receptor tyrosine phosphatase sigma.[183]–[185]

Because a reduction in CS synthesis was expected in T1KO mice, we examined recovery after SCI.[186] Values of the Basso mouse score, which is used to evaluate motor function recovery after SCI in mice,[187] reached as high as 7 after 8 weeks in T1KO mice.[186] To the best of our knowledge, this score in T1KO mice is the highest in mice deficient in a single gene. Histologically, 40-fold more serotonin-positive motor fibers were confirmed in regions distal to the injury compared to wild-type mice.[186]

We suspected that another factor plays a role in enhancing axon regeneration in addition to the reduction in CS. We focused on another glycosaminoglycan, HS, which shares a synthetic pathway with CS, just before the T1 reaction.[188] The effects of HS on neuronal growth are totally opposite to those of CS.[184],[189]–[191] After SCI in T1KO mice, all HS synthesizing enzymes were rapidly upregulated, and the amounts of HS increased by more than 40-fold. Degradation of HS by bacterial heparitinase in injured areas cancelled out the positive effects in T1KO mice. Thus, we concluded that not only a decrease in CS but also upregulation of HS contributes to the marked axon regrowth in T1KO mice. These results suggested that the CS/HS ratio is essential for establishing the most suitable extracellular environment to support axon regeneration[184] (Fig. 7). Including our idea, strategies for regulating CS have been developed to stimulate axonal regeneration after SCI.[179]

Intrinsic inhibitory mechanisms for limiting the maintenance of growth cone activity are present in the adult brain, and approaches for removing these factors have led to partially successful axon regener-
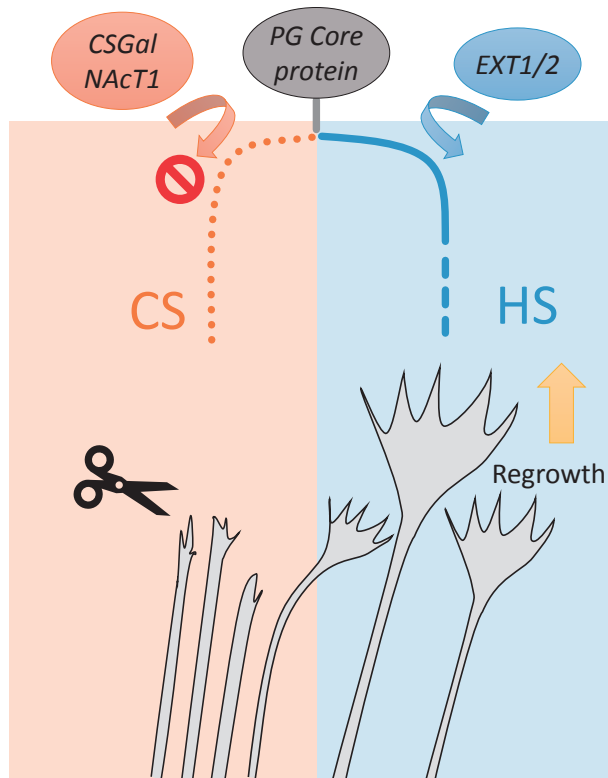
Fig. 7.　Mechanisms of axon regrowth after SCI in T1KO mice. T1KO mice are deficient in T1 expression, which is normally upregulated within 24 h after SCI. CS expression is downregulated by 30%. Simultaneously, HS expression is highly upregulated by 20-fold, which is favorable for axonal growth. Inhibition of the growth cone is reduced, and stimulation of growth is elevated, respectively, and tremendous recovery from SCI occurs.

ation.[192),193)] Such mechanisms are generally energy-consuming, and maintaining them at an adult stage will be disadvantageous for energy economics in the mammalian brain, which has a large number of neurons.

Unsolved: For efficient axon regeneration after SCI, we need to elucidate the mechanisms of transcriptional regulation of glycosyltransferases, and probably sulfotransferases, which are involved in CS/HS synthesis and are thought to be the key processes. Unfortunately, neither the transcription factors nor the promoter structures for these genes are currently characterized, probably because these genes are not believed to be dynamically regulated. However, T1 is usually highly induced within 12 h of SCI,[186)] indicating that the mechanism of CS synthesis should be clarified. CS proteoglycans are involved in psychiatric diseases,[194),195)] and pathogenesis in-

volving nerve growth and synaptogenesis should also be investigated.

8. Conclusions and future perspectives

The following points are important, unsolved problems that are essential for a complete view of the mammalian molecular mechanisms of CNS nerve growth. Most are derived from our new results using novel techniques.

8.1. 3D imaging of each component. Our superresolution imaging revealed the importance of movements in the z-axis direction. Most membrane proteins, including guidance receptors, F-actin, and MTs, likely move three-dimensionally for axonal growth. Testing of most current molecular hypotheses of growth cone behaviors has relied on the results of 2D analysis. 3D analysis may lead to revision of the conclusions.

8.2. The sites of vesicular fusion in the growth cone. Precisely speaking, the vesicular fusion site for axonal growth is not limited to the growth cone area; however, this is the most likely site. Why massive fusion sites have not been identified yet is one of the most important questions. Although some previous reports have described vesicular fusion sites using TIRF microscopy,[196),197)] only a portion of fusion sites for continuous nerve growth have likely been identified. We have partially clarified F-actin-vesicle interactions,[132)] but this is only one clue of the complex cytoskeletal-membrane relationship in nerve growth.[198)–200)]

8.3. F-actin-MT interactions. F-actin is thought to lead the growing and sliding of newly polymerizing MTs into a growth cone when the axon grows. The molecules involved in this process are not clearly understood. As described above, drebrin, MAPs, or MACF[13),15)] have been reported to be involved, but other linkage proteins may exist.

8.4. Cortical F-actin dynamics. The morphology of the growth cone changes to the next, distal axon shaft when the axon grows by sliding of MTs, and this process is dependent upon cortical F-actin dynamics in the C-domain; however, this process is totally unanalyzed. Because membrane skeletal proteins, such as spectrin, ankyrin, and band 4.1 proteins, are enriched in the growth cone,[15)] superresolution analysis of the dynamics of these proteins will be a breakthrough in solving this problem.

8.5. Metabolism and signaling. The growth cone should have features of metabolism due to the presence of metabolic enzymes as revealed by

proteomics. However, no information has been reported concerning the relationship between metabolism and nerve growth signaling.

8.6. Retrograde signaling for nerve growth. Growth cone signaling provides feedback to the cell body, and continuous anterograde axonal transport is needed. However, these processes are mostly unknown in the mammalian growth cone.

8.7. G-protein signaling. Heterotrimeric G-proteins are enriched in the growth cone, as demonstrated by proteomics, but this has been known for more than a quarter of a century. Because the amounts and species of GPCRs are quite few in the growth cone,[13] how G-proteins function in the growth cone is not known.

8.8. Lipid raft signaling. Theoretically, most guidance signaling proteins should be concentrated in lipid rafts, as described above. However, this concept has not been demonstrated directly. Both more efficient systems for visualizing lipid rafts and purer fractionation of lipid raft components should be devised.

8.9. GAP-43. This protein has been studied for more than 40 years, but its roles in the growth cone have not been clarified. Because of the considerable abundance of this protein in growing/regenerating axons and the growth cone, this protein requires additional studies.

8.10. JNK activity. We have shown that JNK activity is indispensable for nerve growth in mammalian CNS neurons. Why and how this protein kinase is activated at developing stages are the next problems to be solved in brain development.

Acknowledgments

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Profile

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