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Review article

Proteomic identification of the molecular basis of mammalian CNS growth cones

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ABSTRACT

The growth cone, which is a unique structure with high motility that forms at the tips of extending axons and dendrites, is crucial to neuronal network formation. Axonal growth of the mammalian CNS is most likely achieved by the complicated coordination of cytoskeletal rearrangement and vesicular trafficking via many proteins. Before recent advances, no methods to identify numerous proteins existed; however, proteomics revolutionarily resolved such problems. In this review, I summarize the profiles of the mammalian growth cone proteins revealed by proteomics as the molecular basis of the growth cone functions, with molecular mapping. These results should be used as a basis for understanding the mechanisms of the complex mammalian CNS developmental process.

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1. Introduction

The growth cone is a highly motile structure that specifically forms in the developing and extending axons and dendrites. Historically, this structure was discovered by a distinguished Spanish anatomist, Santiago Ramón y Cajal, more than a century ago (Cajal, 1890). Then, a cell culture study performed by Harrison revealed that the growth cone actually moves (Harrison, 1910), and a pioneer imaging study by Junnosuke Nakai (Dept. Anatomy, Unit Tokyo at that time) revealed that growth cone movement is regulated by multiple cues (Nakai and Kawasaki, 1959). Since the development of the molecular cell biology of the neuron from a study indicating that F-actin is an origin of filopodial growth (Bray, 1970) and since genetic approaches to neural wiring analysis from 1980 to 2000, knowledge regarding the molecular basis of growth cone behavior has expanded rapidly, including guidance molecule-dependent signaling pathways (Goodman, 1996; Kolodkin and Tessier-Lavigne, 2011). The fundamental molecular mechanisms of most other neural developmental processes, such as neural cell lineage, neurogenesis, neuronal migration, and synaptogenesis, appear to be generally understood even in the mammalian CNS; however, the fundamental molecular machinery for growth cone functions remains poorly understood in the CNS of higher organisms.

Growth cone research has primarily been performed using (1) model organisms, such as *Caenorhabditis elegans* or *Drosophila*; (2) invertebrate neurons, such as *Aplysia*; or (3) peripheral nerves of vertebrates, such as dorsal root ganglion (DRG) neurons of the chick. Most of the information regarding molecular signaling pathways is based on the results from the above specimen. Thus, most of the reviews and the hypotheses have been based entirely or partly on these works. Although these works have certainly greatly contributed to the progress of this field, currently, the most intensively studied growth cone is derived from the mammalian CNS. Clearly, the growth cones of simpler organisms or simpler nervous systems (such as DRG) have completely different molecular compositions and interactions compared with the mammalian CNS; the simple hypotheses constructed using non-mammalian growth cones studies should be examined to determine whether these hypotheses can also be applied to the mammalian CNS.

Because growth cone studies are more difficult than other compartments of the neuron, various findings have been integrated from many types of neuronal growth cones. For example, in the cases of cell biology and imaging, a large size and rapid movements are favorable; thus, the growth cones of chick DRG or invertebrate growth cones from organisms such as *Aplysia* have been frequently used. Pharmacological approaches have also used vertebrate neurons, such as *Xenopus* retinal neurons, chick DRG, and mammalian cell lines. However, in this decade, mammalian CNS neurons, such as cortical or hippocampal neurons, have been utilized to discover additional molecular signaling pathways. Because hippocampal and DRG neurons have been shown to considerably differ from each other (for example, Amin et al., 2013), the molecular mechanisms of their growth cone behavior should also be considerably different.

One technical limitation was that no method was available for determining the numerous protein components in the growth cone of the mammalian CNS. However, proteomic analysis, which quantitatively identifies numerous proteins, has been devised and advanced in a given system, and this method has completely overcome the methodological limitations for determining protein components (Craft et al., 2013). Using this method, we succeeded in systematically profiling important identified components, namely, their sites of the localization, and determined the highly concentrated proteins. In addition, RNAi against many of these genes was an effective method for determining novel important proteins for axonal growth (Nozumi et al., 2009). The combination of our work succeeded in not only finding major components in the growth cone but also postulating the standard methods for profiling the numerous proteins (more than 1000) identified by proteomics.

In this review, I describe the profiles and the putative functions of the proteins identified by our proteomic work in the mammalian CNS.

2. Structure and cellular functional basis of the growth cone

Neuronal processes are classified into axons and dendrites; thus, both axonal and dendritic growth cones are present. However, because the latter are not well characterized, in this review, I only describe the results of the axonal growth cone (when I describe "the growth cone", this term denotes "the axonal growth cone"). In the growth cone, many microtubules and various vesicles are concentrated in the area near distal axonal shafts, which is called the central domain/region (C-domain/region) of the growth cone (Dailey and Bridgman, 1993). These vesicles are most likely primarily anterogradely transported from the cell body along the concentrated microtubules; however, some of these vesicles may be endosomes derived from endocytosis (Falk et al., 2014). These vesicles most likely function as plasmalemmal precursors for axonal growth, although their exact turnover and recycling are not understood (Pfenninger, 2009). Even in the era of electron microscopy, the distribution of organelles in the growth cone has not been clearly described, preventing us from analyzing molecular signaling in the current growth cone studies. In contrast, the area near the leading edge, which is called peripheral domain/region (P-domain/region; Dailey and Bridgman, 1993), has the higher concentration of dynamic actin filaments. In general, the P-domain is more deeply involved in growth cone motility; however, only this domain enables the growth cone to move, and the coordination between F-actin rearrangements in the P-domain with microtubule growth in the C-region is essential to the growth cone function (Dent et al., 2011; see Fig. 1A).

Using pharmacological methods, many studies have examined signaling pathways required for neuronal growth and have

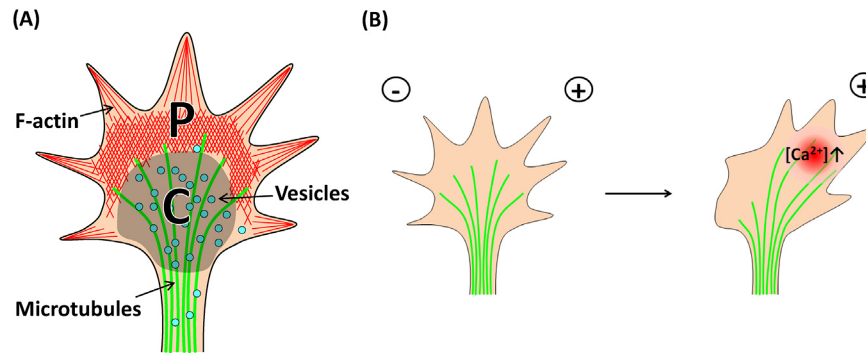

Fig. 1. Classical views of the growth cone. (A) A generalized illustration of the mammalian growth cone. A growth cone is divided into two compartments, namely, the C (central)-domain and the P (peripheral)-domain. F-actin (red) is densely localized as lamellipodia (red plane) and filopodia (red lines) in the P-domain. Vesicles (blue) are enriched in the C-domain along the microtubules (green). (B) Axonal growth, turning and signaling. Axonal growth is directed by the positive (attractive, +) and the negative guidance molecule (repulsive, −) most likely via elevated intracellular Ca^{2+} (or cAMP) concentrations (red). Microtubule polymerization (green) follows this direction.

contributed to identifying signaling molecules regulating growth cone functions (Zheng and Poo, 2007). However, these studies have limitations unless the inhibitors are available.

Many of these studies have indicated that two second messengers are featured in the growth cone, one is Ca^{2+}, and the other is cAMP (Zheng and Poo, 2007; Mortimer et al., 2008; Tojima et al., 2011). These molecules are generally accepted as important signaling molecules, even in the mammalian CNS growth cone; however, the model shown in Fig. 1B is too simple to explain growth cone behavior and should be more complex, because of the proteins related to these processes that we have identified in mammalian CNS growth cones by proteomic methods. I will discuss the proteins involved in regulating these second messengers later.

3. Proteomic analysis strategy for the mammalian growth cone

3.1. *How to analyze the growth cone proteins by proteomic methods*

As I discussed above, to understand the molecular mechanisms of CNS mammalian growth cone functions, we must directly obtain the protein information localized in the growth cone. Current proteomic methods provide quantitative and qualitative information regarding each identified protein (Craft et al., 2013). Thus, proteomic methods are the most suitable methods for our purposes.

The first problem was determining which proteomic method to use. Concerning the proteomic methods, we chose shotgun proteomics (Brusniak et al., 2012). This method was combined with the following analytical steps: (1) fractional proteins were directly catalyzed by trypsin, (2) each peptide fragment was separated by HPLC (high performance liquid chromatography), (3) its sequence was determined by mass-spectrometry; and (4) annotation was performed to determine the protein origin, and quantification was performed using proteomic software. The shotgun method is able to directly analyze many proteins (more than 1000) with only a few treatments.

The next problem was determining how to collect the growth cones. Because each growth cone is small, to collect the growth cones separately is considerably difficult. The other and much larger problem is that the protein abundance is widely varied in each sample. For example, compared with the most abundant protein groups such as tubulin or actin, the functional minor proteins are usually less than 1%. If the amount of the starting material were small, then the proteomic analysis would most likely reveal only the most abundant and widely distributed proteins already known to be localized in the growth cone. Thus, we abandoned cutting the growth cone and used the bulk isolation method to collect growth cones. These methods are based on subcellular fractionation, and from the developing rodent brains, several milligrams of the proteins can be collected (Pfenninger et al., 1983; Gordon-Weeks and Lockerbie, 1984; Gordon-Weeks, 1988), similar to collecting synaptosomal fractions in adult rodent brains. In these methods, the isolated growth cone or growth cone particles (GCPs) are enriched in this fraction, and the hypotonic treatment of this fraction provides the growth cone membrane (GCM; Ellis et al., 1985). These small amounts should be sufficient to discover many important proteins only detected or identified using proteomics. Using these methods, we succeeded in characterizing many previously unknown proteins in the growth cone (Nozumi et al., 2009).

In addition, recently, another group that first devised these fractionation methods provided further GCP proteins identified by proteomics (Estrada-Bernal et al., 2012). Although these authors identified approximately 1800 species, which is nearly twice as many as our results, almost all our results were validated in their report.

3.2. *The validation methods for the proteomic analysis of the growth cone*

For the proteomic analysis of subcellular fractions, the most important concern is the degree of the contamination from other cellular compartments (Drissi et al., 2013). If these fractions are contaminated, then the primary proteins of these fractions should be present in large amounts, and the reliability of the results decreases. To reduce this problem, the purity of a given fraction (in this case, the GCP/GCM fractions) should at least be excessively higher; however, discarding contaminating proteins to maintain high purity reduces the amount of analyzable proteins, and then only proteins present in large amounts are identified.

Pfenninger and his colleagues used general biochemical methods as validation methods (Estrada-Bernal et al., 2012). Specifically, these authors focused on comparing GCP proteins to those proteins appearing in the other fractions. For example, these authors quantitatively measured the contents of several proteins in LSS, nuclear and ER-Golgi fractions, as well as GCPs, and attempted to determine the concentration ratios of these proteins. This method was followed by standard comparative proteomics; however, one problem with this method, which is frequently used in the proteomic analysis of organelles to determine the purity of each fraction and the contamination from the other fractions, is that fractions other than GCPs are less highly purified; thus, biochemical methodology alone is insufficient to judge the purity of GCPs, although biochemical quantitative comparisons using this method provide some important information.

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Table 1
The major growth cone proteins, which are highly enriched in GCP or GCM than adult synaptosomes.

	Gene name		
GCP > synaptosome	Crmp1	Dpysl3	Dpysl5
	Fasn	Hsp90ab1	Map1b
	Tuba1a	Tuba1c	Tubb2a
	Tubb3	Ywhag	Ywhaq
	Ywhaz		
GCM > synaptosome	Ctnna2	Ctnnb1	Cntn1
	Dync1h1	Eef1a1	Gap43
	Gnai1	Gnai2	Gnaq
	Hsp90b1	Lrp1	L1cam
	Myh11	Ncam1	Nefm
	Rtn4	Tuba1b	Tuba3a
	Tubb3	Tubb5	

We performed a different strategy from that used by Pfenninger's group. Because GCPs should represent the morphological growth cones, the proteins identified as GCP proteins by proteomics should be present in the growth cone (Nozumi et al., 2009). Thus, immunostaining the cultured neuronal growth cones was adopted as our validation method (Nozumi et al., 2009). Surprisingly, although we performed immunostaining for more than 200 proteins, no false positive cases, i.e., when only the cell bodies or the axons/dendrites were staining but not the growth cone, were found. These results confirmed the extremely high purity of our GCP/GCM fractions (Nozumi et al., 2009). We summarized the major proteins identified by proteomics, which were more concentrated in GCPs/GCMs than in the adult synaptosomes (Table 1).

4. Neuronal growth-associated proteins: nGAPs

Currently, many active studies of synaptic functions at the molecular level exist. These studies are dependent on the extensive characterization of approximately fifty species of synapse-associated proteins within the last two decades, including their biochemical interactions, localization, and roles in regulating synaptic functions (Bai and Witzmann, 2007; Igarashi and Ohko, 2009; Südhof, 2013). In contrast to synapse-associated protein in the mature synapses, we did not have molecular markers of growth cones, except for a classical neuronal growth-associated protein, GAP-43 (also named neuromodulin; Denny, 2006). If molecular markers are postulated, then growth cone research should develop more rapidly.

Based on our proteomic analysis, I intended to postulate molecular markers of growth cones. For these purposes, I proposed three criteria: presence, concentration, and function. The presence criterion required demonstrating which proteins are present in growth cones before the proteomic analysis and was the most important and difficult criterion. However, now, we have protein data for growth cones. We performed systemic immunofluorescence studies to identify the proteins highly concentrated in the growth cone area. Using this approach, we identified approximately 100 proteins that specifically localized in the growth cone compared with the axon. Finally, using RNAi, we selected the proteins affecting axon growth.

Using GAP-43 as a standard, we performed systematic immunofluorescence studies and verified the proteins that were statistically more concentrated in the growth cone area than GAP-43. We found that more than 70 proteins are more concentrated in the growth cone area and that approximately 30 proteins are equally concentrated in this area.

To systematically approach RNAi, we devised methods using EGFP-transgenic rats. Specifically, we introduced both siRNAs against EGFP and against given genes to the cultured cortical neurons of these rats and selected rats with reduced immunoreactivity of both GFP and the given protein; then, we measured the lengths of these axons (Lu et al., 2008, 2011).

Table 2
Protein categories of nGAPs.

Gene name	Functional categories	Axonal length (%)
Fabp7	Signaling	46.1 ± 5.4
Cotl1	Cytoskeletal	55.3 ± 5.8
Cap1	Cytoskeletal	56.0 ± 7.7
Capzb	Cytoskeletal	60.8 ± 5.0
Sept2	Cytoskeletal	64.5 ± 5.4
Tmod2	Cytoskeletal	64.7 ± 4.9
Gpsn2	Metabolic enzyme	65.1 ± 5.8
Pacs1	Signaling	69.9 ± 6.4
Rtn1	Signaling	71.1 ± 5.5
Strap	Signaling	73.4 ± 4.7
Clptm1	Receptor	75.4 ± 10.9
Cyfip1	GTP binding	78.6 ± 7.9
Snap25a	Membrane traffic	80.5 ± 5.3
Crmp1	Signaling	81.0 ± 2.9
Stx7	Membrane traffic	82.8 ± 5.5
Gnai2	GTP binding	83.8 ± 2.7
Gnao1	GTP binding	83.9 ± 4.2
Farp2	GTP binding	84.2 ± 7.1

See Nozumi et al. (2009) and Fig. 1.

Thus far, by combining these methods, we have identified approximately 20 proteins as functional molecular markers of the growth cone. These proteins are more concentrated or equally concentrated in the growth cone compared with GAP-43, and their decreased expression in this region reduced axon growth. Thus, these proteins are assumed to be essential to nerve growth and to be involved in the functions of mammalian CNS growth cones (Nozumi et al., 2009). We named these proteins neuronal growth-associated proteins (nGAPs). Their categories are widely distributed and include cytoskeletal regulators, vesicular trafficking proteins, signaling molecules, and so on (Fig. 2 and Table 2).

At first, we were slightly surprised that fewer proteins were found compared with those proteins reported to be involved in neurite growth in simpler model organisms (*C. elegans* or *Drosophila*). The reason for this difference is that these proteins are most likely present even in these organisms but are not generally concentrated in their nervous systems in contrast to the mammalian CNS. These results clearly indicate that our proteomic methods are revolutionary for finding new molecules involved in growth cone functions in mammals. In addition, this finding suggests that the molecular mechanisms of mammalian neuronal growth considerably differ from those molecular mechanisms of model organisms. Thus, the mutant studies of *C. elegans* and *Drosophila* will insufficiently provide new findings common to mammalian CNS neuronal growth.

Although these nGAPs were postulated using the cortical neuron, whether these nGAPs are generalized growth cone markers was not clear. We examined this point using PC12D cells, which are closely related to PNS neurons. All of the nGAPs postulated using the cortical neurons satisfied the criteria of "nGAPs", even in PC12D cells, which are distantly related to cortical neurons (Lu et al., 2011). Thus, our discovered nGAPs have been demonstrated to be universal growth cone markers that are not dependent on neuronal species.

Recently, Pfenninger and his colleagues reported the results of their own proteomic studies concerning GCPs (Estrada-Bernal et al., 2012). These authors have identified twice as many protein types as our studies have. The major proteins in their reports were almost identical to those proteins in our results, and these authors have described the more proteins that our groups did not report. However, we confirmed most of our proteins using phosphoproteomic methods we recently began (data not shown). In contrast, these authors did not analyze the GCM, which is critically important

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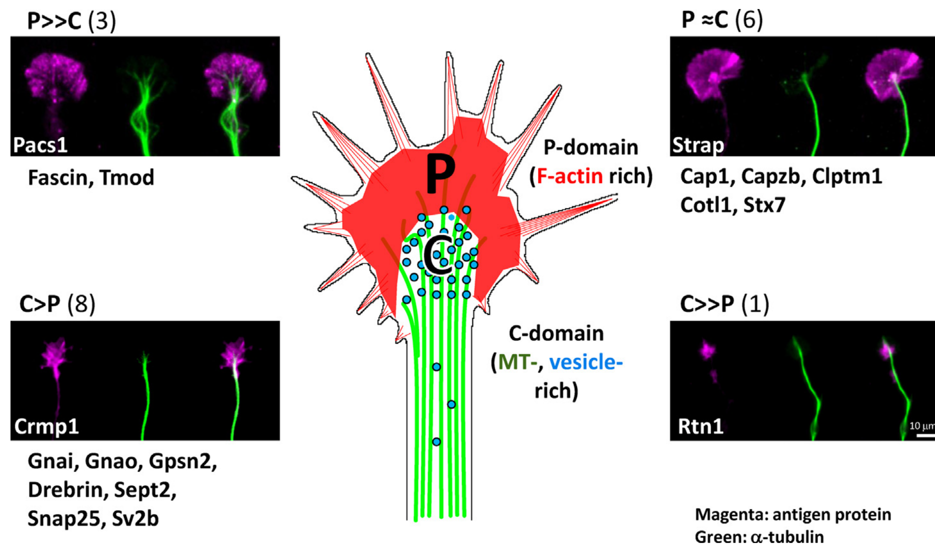

Fig. 2. The distribution of nGAPs in the growth cone. Based on their localization, nGAPs are classified into four groups. (1) The group that is selectively distributed in the P-domain (P ≫ C); (2) the group that is evenly distributed in both domains (P ≈ C); (3) the group that is dominantly distributed in the C-domain (C > P); and (4) the groups that is selectively distributed in the C-domain (C ≫ P). The number in parenthesis represents the total number of identified peptides derived from a given protein. See Fig. 1A and Table 2.

growth cone signaling. Regarding membrane proteins, differences in abundance exist between their protein data and ours because membrane components occupy only a small portion of the GCPs.

5. Characteristics of the growth cone proteins identified by proteomics and the molecular anatomy of the growth cone

5.1. Cytoskeletal proteins

5.1.1. MTs and MT-related proteins

After the direction of the growth cone advance, as described above, MT sliding into the growth cone and its stabilization are the processes by which the growth cone portion changes to the distal axonal shaft (Dent et al., 2011). Concerning MT stabilization, microtubule-associated proteins (MAPs) are bound to MTs. Because of this stabilization, MTs can grow long within the neuron. The growth cone MAPs are primarily composed of MAP1B and tau proteins, which are regulated by phosphorylation. +TIPs are a completely different protein group that selectively binds to MT (+)-ends at the selective polymerization site. +TIPs are composed of many types of subgroups, each of which are structurally different (Akhmanova and Steinmetz, 2010).

As we described above, MTs are the necessary structures for axon growth and formation on the largest scales; thus, the most abundant protein found in the growth cone by proteomic methods is most likely tubulin. The mutation of one of the isoforms found in the growth cone by proteomics, TUBB3 (β3), has recently been reported as a cause of the human hereditary disease of axon guidance (Tischfield et al., 2010). Among the microtubule-associated proteins (MAPs), which induce stable polymerization of MTs for long MT growth, the most abundant one is MAP1B and then tau proteins. MAP1B and tau are classically known to be important for axonal growth and are highly phosphorylated (Riederer, 2007). Interestingly, these proteins are differently regulated by phosphorylation; MAP1B phosphorylation is enriched in the growth cone and in the distal axon, whereas tau is distributed by a controversial gradient (Mandell and Banker, 1996). Additionally, minor MAPs, such as MAP1A and MAP6 (STOP) proteins, have been identified.

In contrast, +TIPs in the growth cone were not well characterized before our work. We found several proteins belonging to +TIPs, including EB1, EB3, and APC2. Using real-time imaging, we have confirmed that EB3 is bound to MT (+)-ends in the growth cone. The contribution of APC2 in neuronal growth was recently shown by Noda and his colleagues using chick retinal projection systems (Shintani et al., 2009). Additionally, interactions between classical MAPs and +TIPs, for example, the interaction between MAP1B and EB1/3, were reported to regulate axonal microtubule growth (Tortosa et al., 2013). CLASP2 was also identified in the GCM and recently characterized as a regulator of axon growth and polarity formation (Hur et al., 2011; Beffert et al., 2012; Lewis et al., 2013; see Table 3).

Two MT-dependent motors exist, i.e., retrogradely moving dynein and anterogradely moving kinesin (Vale, 2003; Suter and Miller, 2011; Prokop, 2013). Our proteomic study clearly revealed that dynein is present in an overwhelmingly larger amount than each kinesin species in GCPs. Concerning neurons, at least one of the dynein-dependent cargoes or molecules related to neurons are signaling endosomes or their related proteins, which are involved in signaling to the nucleus (Fainzilber et al., 2011). Dynein is also thought to interact with actin-binding proteins and other MT-binding proteins to extend the axon (Myers et al., 2006; Grabham et al., 2007). We also detected the dynactin complex components related to retrograde axonal transport, such as dynactin 1 and ARP1 in the GCP. We recently found that some of the vesicular movements are retrogradely moving and rapid using a live-imaging method (M. Nozumi and M.I., unpublished results). These movements may be dynein-dependent and may have some roles in growth cone functions (Abe et al., 2008).

Concerning kinesins, more than 40 kinesin family members are known to correspond to the distinct cargoes (Vale, 2003). However, each kinesin family member is most likely not as abundant in the growth cone as dynein; for example, the most abundant KIF5 protein, which carries the slow axonal transport cytoskeletal proteins, was less abundant in the growth cone.

Several of the stathmin family members, which are MT-depolymerizing factors, were found. SCG10/stathmin-2 is a famous growth-associated protein (Riederer et al., 1997; Lutjens et al., 2000; Grenningloh et al., 2004; Togano et al., 2005), and stathmin and RB3 have been identified by our approach. The major differences between stathmin and SCG10 are that the former is cytosolic and that the latter is membrane-bound by palmitoylation. Thus, SCG10 is enriched in the GCM (Nozumi et al., 2009).

Table 3
Localization of cytoskeletal, trafficking, and signaling components within the mammalian CNS growth cone.

Gene name	Distribution
Cytoskeletal	
4.1N	Actin bundles
Ank2	Plasma membrane
Arp1	C-domain
Arpc1	C-domain
Arpc5	C-domain
Cap1	C-domain
Capzb	C-domain
Cofilin	C-domain
Cotl1	C-domain
Drebrin	C-domain
Fascin	Actin bundles
Lasp	C-domain
Macf1	Actin bundles
Myo5a	C-domain
Tmod	Actin bundles
Tmsb4x	C-domain
Membrane traffic	
Ap180	P-domain
Ap2a1	Plasma membrane
Ap2b1	P-domain
Cltc	C-domain
Dynamin	Plasma membrane
Munc-18	C-domain
Pclo	C-domain
Picalm	Plasma membrane
PSD-93	P-domain
Scamp	C-domain
Snap25	C-domain
Snap29	C-domain
Stx1	C-domain
Stx16	C-domain
Stx7	Plasma membrane
Stx8	C-domain
Sv2a	C-domain
Sv2b	C-domain
Syn2	C-domain
Syt	C-domain
Vamp	C-domain
Signaling	
14-3-3e	Plasma membrane
Arvcf	C-domain
Caspr2	C-domain
Crmp1	C-domain
Crmp2	C-domain
Crmp3	C-domain
Crmp4	C-domain
Crmp5	C-domain
Ctnna1	Plasma membrane
Ctnna2	C-domain
Fabp5	C-domain
Fabp7	C-domain
Gap43	C-domain
Marcksl1	P-domain
Ncdn	P-domain
Ndrg1	C-domain
Pacs1	P-domain
Palm	C-domain
PAR3B	C-domain
Ppfia1	Plasma membrane
Rtn1	C-domain
Rtn4	C-domain
Strap	C-domain
Tks5	C-domain

MT-depolymerization factors are believed to increase the dynamic instability of MTs in the growth cone.

5.1.2. Actin filaments and actin-binding proteins

As described previously, filopodia and lamellipodia in which the dynamic actin filaments are concentrated in P-region are present in the growth cone. The actin polymerization and depolymerization cycle is believed to determine the direction of axon growth (Dent et al., 2011). We will discuss the actin-binding proteins identified by proteomics. Additionally, a cortical actin structure exists in the C-region. These structures are the membrane skeletons that exist directly beneath the plasma membrane. Clearly, the P-domain actin filaments are extremely dynamic, and the C-domain actin filaments are thought to be relatively stable. The functional importance of cortical actin in growth cone functions is poorly understood, although MT sliding toward the growth cone is likely the essential mechanism for axon growth, and at this step, dynamic deformity of cortical skeletons is required.

Concerning the P-domain actin-binding proteins, myosins and ARP2/3 family members are the most important components. The ARP2/3 complex is also important for the nucleation of actin and for the physiological formation of F-actin (Goley and Welch, 2006). Additionally, using *Aplysia* and cell lines, the ARP2/3 complex has been shown to be responsible for filopodial formation and the dynamic actin network in the P-domain (Korobova and Svitkina, 2008; Yang et al., 2012). ARP2, ARP3, ARC1, and ARC5 were detected in the proteomic results (see Fig. 2); the ratio of actin to the ARP2/3 complex is most likely 10:1. In our preliminary live-imaging results, ARP3 was found to be closely related to the behavior of actin compared with ARP2, indicating that these components do not always move together.

Myosin II, which is the conventional myosin, is believed to contribute to the clutch functions of actin filaments in the growth cone (Jay, 2000). Our proteomic study revealed that myosin II is the most abundant actin-regulatory proteins and, remarkably, that this protein is primarily membrane-bound. Thus far, the contribution of the membrane in the clutch roles has not been sufficiently considered. Our results suggest that the interaction between myosin II and membranes regulates F-actin.

Myosin-V is a featured molecular actin-based motor, which moves anterogradely along F-actin conveying various cargoes, such as ER and synaptic vesicles (Vale, 2003). In our studies, myosin-V has quantitatively been shown to be an important GCM protein. If axonal growth requires actin-based vesicular transport in growth cones, then myosin-V should be required for this event. In addition, some reports have indicated that myosin-V is involved in filopodial activity (Wang et al., 1996; Tamada et al., 2010); thus, the exact localization of myosin-V is important for the next step.

For the former categories of actin-binding proteins, cofilin (also called actin depolymerizing factor (ADF); Sarmiere and Bamburg, 2004), a G-actin-binding capping protein, is the most abundant protein, followed by fascin, which bundles F-actin (Jayo and Parsons, 2010). Mizuno and his colleagues proposed that the capping activity of cofilin is regulated by LIM kinase-dependent serine 3 phosphorylation. This phosphorylation inactivates the effect of cofilin on actin (Yang et al., 1998). Recently, using cofilin-KO mice, this protein was suggested to mediate the retrograde flow of actin, as well as of myosin-II and the ARP2/3 complex (Flynn et al., 2012).

CAP1 is also a G-actin-binding protein (Hubberstey and Mottillo, 2002) and one of nGAPs. Using a genetic approach in *Drosophila*, CAP1 was found to be involved in axon guidance; however, its cellular mechanism remains unclear (Wills et al., 2002). We revealed that CAP1 overexpression and RNAi induced changes in the growth cone size and in the growth activity, indicating that CAP1 modulates the growth cone function by modifying the P-domain actin (Nozumi et al., 2009; Lu et al., 2011).

Drebrin has two isoforms (Shirao and González-Billault, 2013); drebrin-A is known to be a marker of the dendritic spines; however, drebrin-E is thought to be a marker of axon growth, and we identified this marker as a GCP actin-binding protein (Nozumi et al., 2009). This protein was reported to interact with EB3 and to be a possible linker between MTs and F-actin (Geraldo et al., 2008).

Regarding the cortical actin-binding proteins in growth cones, the molecular information for these proteins was less known before our work. We identified large amounts of spectrin, ankyrin, band 4.1, and tropomodulin (Table 3); thus, the components of the cortical skeleton (or membrane skeleton; Baines, 2010) are extremely important to the growth cone functions (for example, a *Drosphila* paper (Hülsmeier et al., 2007)). Because the MTs slide into the growth cone C-domain and because this portion should become the next distal portion of the axon, the deformity of these cortical skeletal components remains one of the unsolved problems for axonal growth mechanisms.

5.1.3. Other cytoskeletons

Regarding the components of intermediate filaments (Lépinoux-Chambaud and Eyer, 2013), aside from the neurofilament triplet proteins, α-internexin (class IV intermediate filament protein) is quite abundant. Morphologically intermediate filaments were not found in the growth cone; thus, these components are unlikely to be polymerized forms (Liem and Messing, 2009). If so, then the structure that these components take is an interesting problem.

Septin, which is considered the fourth component of the cytoskeleton, is composed of the heterotrimeric unit in a GTP-dependent manner (Barral and Kinoshita, 2008). Septin 2, which is one of the components, is also an nGAP (Nozumi et al., 2009). A decade ago, a report suggested the involvement of this molecule in neurite growth using PC12 cells (Vega and Hsu, 2003); however, our results clarify this point.

Spectraplakins (MACF1) are a molecular family of proteins with large molecular masses that link F-actin and MTs. MACF1 is bound to F-actin in its N-terminus and is linked with MTs via EB1 in its C-terminus. As a result, this molecule promotes MT-mediated axonal growth (Alves-Silva et al., 2012).

5.2. Vesicular trafficking proteins

Electron microscopy (EM) studies have revealed that many vesicles accumulate in the C-region of the growth cone (Dailey and Bridgman, 1993). However, several classical EM analyses have reported regarding the various types of vesicles in this region. Because these vesicles were classified only by their EM morphologies, whether each vesicle is actually distinct from each other is unknown. The “growth cone vesicles” are more electron-lucent and larger than synaptic vesicles; thus, these vesicles completely differ from each other (Pfenninger et al., 1983; Igarashi et al., 1997). As I demonstrated previously, at least some of these vesicles are used for vesicular fusion-dependent plasmalemmal expansion through exocytosis, and this process is performed by the SNARE mechanism (Igarashi et al., 1996). This mechanism is also utilized by mature SVs (Igarashi and Watanabe, 2007; Igarashi and Ohko, 2009; Südhof, 2013), although the regulation mechanisms of these processes should differ from each other (Igarashi et al., 1997).

5.2.1. Proteins involved in exocytosis

Our group and others have shown that nerve growth is performed by the SNARE proteins and their related proteins (Osen-Sand et al., 1993; Igarashi et al., 1996, 1997; Kabayama et al., 2011). These proteins are most likely involved in vesicular fusion and targeting in the growth cone (Pfenninger, 2009). The underlying data were found by proteomics, namely, that these proteins are present in large amounts.

Among the GCM proteins involved in vesicular trafficking, the most abundant one is Munc18-1 (Südhof, 2013). Although this protein is structurally a cytosolic, syntaxin-binding protein, Munc18-1 is highly concentrated in the GCM, suggesting that this protein is bound to syntaxin or to the SNARE complex. Munc-18-1 is much more abundant in the growth cone than syntaxin-1. One report indicated that Munc-18-1 is involved in growth cone morphology (Broeke et al., 2010).

In contrast, the tethering complex, which tethers the vesicles to the plasma membrane before docking (Hertzog and Chavrier, 2011), is much less abundant in the growth cone than the SNARE proteins. For example, rsec6/8, which were once involved in neuronal growth (Vega and Hsu, 2003), were barely identified in GCPs or in the GCM. Although these proteins were recycled and functioned, these proteins should have been more abundant for vesicular fusion, enabling nerve growth. Taken together, the physiological significance of the tethering complex in nerve growth, at least in the cortical neuron, is much less than previously expected, or the tethering mechanism in the growth cone may differs from that of the rsec6/8 complex (Watanabe et al., 2005; Kawase et al., 2006).

Furthermore, synaptic vesicle proteins, such as synapsins, synaptotagmin, synaptophysin, and SV2, have been identified in the GCM (Table 3), and one SV2 isoform belongs to nGAPs (Fig. 2). Although knockout mice of these proteins did not show the phenotype of abnormal axonal growth (Südhof, 2013), these proteins may be involved in fine-tuning axonal growth or pathfinding with molecular redundancy.

SEC22 vesicle trafficking protein-like 1 has been identified, and this ER-resident SNARE protein is much less abundant than plasma membrane SNAREs. Recently, a report suggested that SEC22 is involved in axonal growth via lipid transfer between the ER and the plasma membrane (Petkovic et al., 2014).

5.2.2. Endocytosis-related proteins

Several types of endocytosis exist (Conibear, 2010). Clathrin heavy chains identified by proteomics are much more abundant compared with other endocytic proteins, indicating that clathrin-dependent endocytosis is the primary pathway of membrane vesicular recycling in the mammalian CNS growth cone, where endocytosis is known to be extremely active, as determined by classical EM studies (Winckler and Choo, 2011).

Most of the synapse-dependent endocytic regulatory proteins are not found in the growth cone; the only related and identified GCP/GCM proteins are dynamin I and endophilin B1. This finding is most likely because the endocytosis in growth cones is not necessarily speedily done and because presynaptic endocytosis, where the membrane conductance represents the membrane surface area, is strictly maintained by Ca^{2+} signaling. In addition, dynamin I has also been reported to act as a regulator of nerve growth independent of endocytosis (Yamada et al., 2013).

5.2.3. Rab family proteins

Rab family members are involved in the given vesicular and organelle trafficking (Jordens et al., 2005) and are the most abundant small GTP-binding proteins even in the GCM. Except for rab11 and other members involved in the recycling endosomes, surprisingly, only a few reports directly relate the functions of rab family proteins and axonal growth. Large amounts of and many types of rab family members in the growth cone were identified by proteomics, even compared with the mature synapse, suggesting that uncharacterized, various rab-dependent vesicular transport systems support axon growth or guidance.

Recently, Holt and her colleagues revealed that rab5 and rab4, both of which are endocytic markers of endosomes, are crucial for axon growth in the *Xenopus* retinal axon (Falk et al., 2014). These proteins are also abundant in the GCM of the rat, indicating that these principles are mutual; however, these proteins are most likely modified in the mammalian CNS.

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5.3. Signaling proteins

5.3.1. Receptors

GCM proteomics succeeded in identifying the major receptors (see Table 4). Receptors for axon guidance molecules or neurotrophins of the GCM have also been identified using proteomics, for example, DCC (for netrin), plexin or neuropilin (for semaphorins), and Eph receptors as axon guidance receptors (Kolodkin and Tessier-Lavigne, 2011). Some neurotransmitter receptor proteins have been identified; however, their roles in axon growth and guidance have not been clarified. We found only a few species of GPCRs, including CXCR4, mGluR5, and $GABA_B$ receptors, in the GCM.

Teneurin, which is a receptor-type protein with a higher molecular weight, is a member of the teneurin family. This molecule is widely expressed from the simpler model organisms (*C. elegans* and *Drosophila*) to vertebrates (Kenzelmann and Chiquet-Ehrismann, 2007). The functions of this molecule in development are primarily mediated by hemophilic binding. In particular, this molecule has been shown to have an important role in olfactory connections (Hong et al., 2012). Our proteomic analysis of cortical growth cones identified this molecule and found that this molecule is relatively abundant, suggesting that this molecule may be widely involved in axon guidance (Silva et al., 2011; Young et al., 2013), including cortical neurons.

Concerning synaptogenic receptors, it is reasonable that neurexins have been identified in the GCM because of their future role in synaptogenesis when the growth cone will change to the presynaptic terminals (Silva et al., 2009; Bottos et al., 2011; Bang and Owczarek, 2013). However, their counterparts, neuroligins, have also been identified in the GCM. Because neuroligins are widely distributed and because their interactions are not limited to trans-binding to neurexins, the roles for neuroligins in the growth cone should be investigated. In addition, proteomic methods have identified latrophilins in the GCM. These seven-pass transmembrane proteins, which potentially act as GPCRs (Silva et al., 2009), are receptors for α-latrotoxin, which forces induction of transmitter release and which are not known to be directly related to nerve growth. However, latrophilin (or CIRL) was found to bind to RPTPσ (discussed below as a molecule involved in nerve growth inhibition), suggesting a putative role of latrophilin in growth cone behavior (Krasnoperov et al., 2002). In addition, latrophilin reportedly binds to a teneurin family molecule (discussed above), suggesting its physiological importance for axon growth or guidance (Silva et al., 2011).

Lipoprotein-related protein 1 (LRP1), which is a receptor with a single transmembrane domain and which is involved in endocytosis, is one of the most abundant receptors in the GCM. This molecule may be linked to neurotrophin-Trk signaling (Shi et al., 2009). In addition, LRP1 has been reported to be one of the receptors for myelin glycoprotein (MAG), which is an inhibitor of axon growth (Mantuano et al., 2013).

5.3.2. Heterotrimetric G proteins (G proteins)

G proteins have been thought to be enriched in the growth cone, and our results clearly demonstrated that Go, Gi, Gq, and Gz are present in large amounts in the GCM and are thought to be the most important signaling molecules in the GCM. However, a large discrepancy was also found because the GPCRs, which are most likely coupled with these molecules, were quite less abundant than G proteins, as I mentioned above. Previously, several groups, including ours, showed the involvement of G proteins in growth cone signaling (Strittmatter et al., 1990; Igarashi et al., 1993, 1995; Yuyama et al., 2007; Xiang et al., 2002). However, determining their action modes will require further investigation. In particular, XLas, which is a long form of Gs, was identified in the GCM, and its function should be interesting.

Recently, each G protein (Gs, Gi, or Gq) signal was independently stimulated in neuronal cell lines for neurite outgrowth using optogenetic tools coupled with artificial GPCRs (Karunarathne et al., 2013). This finding is consistent with our proteomic results that such G proteins are quite abundant and important in the growth cone. In addition, recently, intracellular G proteins have been reported to have unique functions in other cells (Lohse et al., 2013), suggesting that these proteins may have new roles in the growth cone if they have such localization sites.

5.3.3. Small GTP-binding proteins (Rho and Ras family proteins)

5.3.3.1. Rho family. Rho family members are known to regulate actin polymerization; thus, these proteins are closely related to the growth cone function (Hall and Lalli, 2010). In particular, cdc42 and rac1 have been demonstrated to regulate ARP2/3-dependent actin nucleation in filopodia and lamellipodia, respectively (Hall and Lalli, 2010). The typical members of the rho family, i.e., rho, rac, and cdc42, are clearly present; however, these proteins are generally present in their membrane-bound forms, suggesting that these proteins are localized as active forms. Among these members, rac1, rac3, rhoA2, rhoC, cdc42, and TC10 are abundantly present. TC10 has been shown to have a relation to the exocyst complex (tethering complex; see Section 5.2; Kawase et al., 2006); however, the difference in their abundances is large, suggesting that a classical tethering mechanism is likely in the growth cone, as I referred to in Section 5.2.

5.3.3.2. Ras family. Ras family members are thought to be involved in adapter proteins in many signaling pathways, including those pathways in the neuron (Hall and Lalli, 2010). Classical "ras" family molecules, such as K-ras, H-ras, N-ras, and novel members, such as ral-A, rap2, rap1, and R-Ras, were identified in large amounts, indicating that ras family members are physiologically important. Thus far, except for the downstream signaling of neurotrophins using PC12 cells, ras family signaling for nerve growth has not been well characterized.

5.3.3.3. Other molecules related to small G protein-dependent signaling. Guanine exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) are known to regulate small G proteins. Among these molecules, GEFs were not abundantly present, and FARP2 (Kubo et al., 2002) and Trio were identified (Briançon-Marjollet et al., 2008). These findings were most likely because many GEFs exist and because each GEF is present in only a small amount but has high activity for small G proteins. Trio is a rac-GEF molecule involved in axonal growth in *C. elegans* and *Drosophila* and may be one of the downstream signals for netrin (Briançon-Marjollet et al., 2008). The most abundant small G protein regulators are GDIs, which maintain their GDP forms and retain these proteins in the cytosol. The rab or rho GDIs are abundant in the GCPs but not in the GCM. One report indicated that rab-GDI is involved in axon membrane trafficking during polarity formation, suggesting that GDIs are involved in axon growth (Wang et al., 2011). Additionally, we identified rab11FIP, which is an adapter protein for rab proteins.

Singar1/RIPX, which is an adapter protein that shows promiscuous binding to multiple small G proteins, is one of the abundant proteins downstream to these small G proteins (Kitagishi and Matsuda, 2013). This protein was first identified by another proteomic study examining axon polarity (Mori et al., 2007).

We also identified *ran*, which is a small G protein involved in nuclear protein transport, and its binding protein, importin-β. We do not think that these proteins are contaminants because of the immunofluorescence results and believe that these proteins

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Table 4
Receptor protein identified in GCM.

Guidance molecule receptors								
Plexins								
Plxna1	Plxna2	Plxna3	Plxnc1					
Neuropilins								
Nrp1	Nrp2							
Eph receptors and ephrins								
Epha3	Epha4	Epha5	Ephb1	Ephb2	Ephb3			
Neurotransmitter receptors								
Glutamate receptor								
Gria1	Gria2	Grid1	Grik1	Grin2b	Grm3	Grm5		
GABA receptor								
Gabra1	Gabra2	Gabra3	Gabrb1	Gabrg2				
Other known receptors								
LDL receptor								
Lrp1								
Trophic factor or growth factor receptors								
Cntfr	Glg1	Igf2r	Insr	Leprotl1	Nptn	Ntrk2		
Neurexins, latrophilins, and neuroligins								
Lphn1	Lphn2	Lphn3	Nlgn1	Nlgn2	Nlgn3	Nrxn1	Nrxn2	Nrxn3
Other receptors								
App	Astn1	Clptm1	Tenm2					

are involved in the long-distance transport of proteins from the growth cone to the nucleus (Yudin and Fainzilber, 2009; Rishal and Fainzilber, 2014).

5.3.4. Protein kinases and phosphatases

5.3.4.1. Serine/threonine kinases. The most abundant protein kinases (S/T kinases) are protein kinase A (cAMP-dependent protein kinase; PKA) and Ca^{2+}/calmodulin-dependent protein kinase (CaMKII). Regarding proteomic aspects, the cAMP theory that this molecule is an important 2nd messenger regulating the growth cone behavior in response to guidance cues (Tojima et al., 2011) appears highly reliable when the signaling is via PKA. CaMKII, which is a major protein for neural plasticity in the mature synapse, is not reported to have a significant role in axonal growth or guidance (Wen et al., 2004), except in *Xenopus*. This finding may not be surprising because the amount of CaMKII is considerably variable depending on the neuronal species (Bolsover, 2005); thus, it is likely that the PNS neurons and neuronal cell lines, which are usually used for neuronal cell biology studies, do not have a large amount of CaMKII.

As for other S/T kinases, PKCβ, casein kinase, and p21-kinase have been identified. Because protein kinase have high potencies to phosphorylate the substrates once activated, most likely, not only the absolute amounts but also the activation processes should be related to the physiological importance; thus, many types of kinases have not been identified.

Although other kinases involved in neural development, such as ERK or GSK3β, are at much smaller amounts than PAK or CaMKII, notably, for these kinases, their physiological activities are more important when activated than their absolute amounts present.

5.3.4.2. Tyrosine kinases. Non-receptor type tyrosine kinases are the signaling molecules for growth regulators studied since the 1980s because the highest expression of *src* and its relative molecules *fyn* and *yes* was observed in the developing mammalian CNS neurons. Our proteomic research revealed the presence of these molecules, although these molecules were less abundantly present than expected. These molecules were studied downstream of IgSFs, which are cell adhesion molecules, such as N-CAM or L1 (Ignelzi et al., 1994). However, the clear involvement of these kinases in growth cone functions has not currently been obtained from the results of knockout mice. These results suggest that IgSFs have different signaling pathways from those pathways regulated by non-receptor tyrosine kinases.

5.3.4.3. Protein phosphatases. Except for some members, most of the phosphatases in growth cones have been not intensively analyzed most likely because the studies of protein phosphatases have generally been delayed compared with those studies of protein kinases. Our proteomic results indicated that each of the S/T phosphatases, i.e., PP1, PP2A, PP2B, and PP2C (=PP3), is present in a given amount in the growth cone (Bolsover, 2005); however, their roles and localization are not precisely known. In contrast, neurobiologists are interested in tyrosine phosphatases because the homologs of these phosphatases are responsible molecules for axon guidance mutants in model organisms (Prakash et al., 2009). In particular, LAR is an important molecule involved in inhibitory guidance. Recently, RPTPσ was identified as a receptor for chondroitin sulfate (Shen et al., 2009), which is a potent extracellular inhibitor of the growth cone.

5.3.4.4. Adapter molecules and other related proteins. The distributions of these proteins are summarized in Table 3.

(a) *CRMP family*: The CRMP family includes the most important and abundant molecules in this group. This family has five members (CRMP1-5), and each of these proteins is abundantly present in the GCPs/GCM. CRMP2 functions in axon formation by stimulating axonal MT polymerization (Inagaki et al., 2001). Kaibuchi and his colleagues have shown that CRMP2 is a multi-adapter protein related to several events required for growth cone behaviors (Nishimura et al., 2005). CRMP4, which is the next abundant molecule, has also been shown to have a role in axon growth. This molecule has also been reported to interact with the 14-3-3 family (see below).

Because the first CRMP family member found in mammalians was a semaphorin signaling transducer (Goshima et al., 1995), each member is thought to be involved in the various signaling pathways of the growth cone. Notably, the *C. elegans unc-33* mutation, with abnormal axon formation and pathfinding, is caused by a CRMP homolog. CRMP5 (CRAM) is primarily associated with mitochondria, and this molecule has been reported to have a modularity role in nerve growth. CRMP5 is also bound to tubulin and CRMP2 but has an opposite effect to that of CRMP2, namely, CRMP5 inhibits axonal growth (Hotta et al., 2005). In

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addition, Kaibuchi revealed that CRMP-2 interacts with Sra-1/CYFIP, which is a rac-associated regulator of the cytoskeleton, and regulates axon formation (Kawano et al., 2005). Because CYFIP is one of the nGAPs (see Table 2; Nozumi et al., 2009), this report is quite interesting.

(b) *Catenins*: Catenins are known components of the membrane skeletal structure in cellular adherens junctions (Maiden and Hardin, 2011), and β-catenin has other functions as a transcription factor during development. In the GCM proteomic results, *N*-catenin (α2-catenin) was found to be quite enriched in the GCM identified by our proteomic studies, and α- and β-catenins were present in given amounts. In the mature synapse, catenins have roles in synaptogenesis and synaptic stabilization; however, notably, these components are abundantly present in the GCM. Currently, the roles of these catenins in nerve growth are unclear. The unbalanced distribution between cadherins (less abundant) and catenins suggests that catenins in the growth cone play other roles in addition to cell adhesion. Nelson et al. showed that α-catenin has a cytoskeletal modulator role based on its actin-binding activity (Yamada et al., 2005), although whether α-*N*-catenin has an activity similar to its isoform is unknown.

(c) *14-3-3 family members*: The 14-3-3 family is known to include adapter proteins for proteins phosphorylated by S/T-kinases (Bustos, 2012). Among the many members of this family, we confirmed that ζ, γ, θ, ε, η, and β forms are abundantly present in GCPs. Since our results were published, some groups have shown that 14-3-3 isoforms are involved in axon guidance signaling. Recently, some groups reported that cAMP-dependent growth cone behavior is regulated by 14-3-3 isoforms downstream of PKA that act as adapter proteins (Kent et al., 2010; Yam et al., 2012). In particular, Founier and her colleagues showed the importance of these proteins by proteomic analysis (Kent et al., 2010). Generally, 14-3-3-isoforms may widely act as adapter proteins of phosphoproteins; whether the phosphorylation of phosphoproteins is closely linked with these molecules should be the next problem.

(d) *Other signaling molecules*: Classical molecular marker of the growth cone, such as GAP-43 (neuromodulin; Denny, 2006), MARCKS protein (Arbuzova et al., 2002; Gatlin et al., 2006), CAP-23 (NAP-22; Maekawa et al., 1993; Caroni, 1997), and paralemmin (Kutzleb et al., 1998) have been identified as the highly concentrated proteins in the GCM. These proteins are also known to be susceptible to in vitro PKC substrates (Mosevitsky, 2005; Denny, 2006) and to be membrane–bound by lipid modifications (palmitoylation and myristoylation; Denny, 2006; Kang et al., 2008). All of these proteins have been shown to be related to axon growth, regeneration, and sprouting. Notably, these molecules are vertebrate-specific.

Phosphoproteomics is a novel proteomic method that comprehensively identifies the phosphorylated sites of the proteins present in a given system. Recently, by applying phosphoproteomics to the growth cone, we found that these PKC substrates are highly phosphorylated in the GCM fractions but that these substrates are not phosphorylated in vivo by PKC but by other kinases because these phosphorylated sites differ from the PKC-dependent phosphorylated sites. The next step of phosphoproteomics is to find the physiological significance of these novel phosphorylation sites.

5.4. Cell adhesion molecules (CAMs)

There are many lines of evidence indicating that the immunoglobulin superfamily cell adhesion molecules (IgSFs) are involved in the growth cone functions in the classical neuronal cell culture studies. However, judging from the analyses of their knockout mice, the lack of any single IgSF member did not cause a large disturbance of axon pathways or of growth cone behaviors, leading to the "dead rock" in the studies of IgSFs focusing the growth cone (Pollerberg et al., 2013). Our proteomic studies revealed that classical IgSFs, such as N-CAM, L1, or contactin, are quite abundant in the growth cone and that IgSFs are clearly the most abundant membrane molecules in the growth cone. As shown in Table 5, more than 20 species of IgSF members were found in the GCM. We also confirmed the presence of at least two species of IgSFs, including IgSF4 (=SynCaM). In contrast, cadherin group members are present but are much less abundant than the IgSFs. As I mention later, notably, catenins, which are intracellularly bound to cadherins, are much abundant than cadherins. IgSFs were the first characterized axon guidance molecules discovered in *Drosophila* (Goodman, 1996). These molecules are also considered the molecules that assure the variety and specificity of synaptic connections (Hattori et al., 2008; Yamagata and Sanes, 2012). These findings suggest that the large amount of IgSF molecules in the growth cone may assure synaptogenesis for not only axon growth or guidance.

In contrast, cadherins are much less abundant than IgSFs. As I mention below, in contrast, catenins that are intracellularly bound to cadherins are extremely abundant in GCPs and in the GCM, and this fact is extremely interesting. For example, TAG-1, which is an IgSF, was recently revealed to be involved in polarity formation (Namba et al., 2014), indicating that abundant IgSFs play a role in axon determination and growth at a given stage.

Table 5
The immunoglobulin superfamily (IgSFs) identified in GCM.

Category	Gene name		
I	Cntn1	L1cam	Ncam1
II	Alcam	Bsg	Cd200
	Cadm3	Cxadr	Igsf3
	Lsamp	Ncam2	Nfasc
	Negr1	Ntm	Opcml
III	Cadm1	Ctcn2	Emb
	Igsf21	Chl1	Nrcam
	Nptn	Pvrl1	Thy1

Groups I, II, and III are classified by the identified peptide numbers (I: more than 10; II; 5 to 2; III: 1).

5.5. Protein translation machinery

Recently, many reports have indicated that protein translation mechanisms are involved in various cellular regulatory processes. In neurons, dendritic spines and postsynaptic regions perform local protein synthesis for synaptic plasticity expression (Bramham, 2008). In contrast, axons and mature presynaptic terminals lack ribosomes; therefore, protein translation never occurs in axons and presynaptic terminals. However, the growth cone has been shown to function in locally synthesizing proteins to respond to guidance molecules; as a result, the direction of growth cone advance changes (Shigeoka et al., 2013). Thus, ribosomes should be present in the growth cone. Our proteomic results clearly demonstrated the abundance of ribosomal components. In addition, the presence of the initiation and the elongation factors for translation has been demonstrated in the growth cone. Local protein synthesis has been shown to be involved in growth cone turning or in its regulation at an intermediate target, depending on the guidance molecules (Campbell and Holt, 2001; Brittis et al., 2002), but not in nerve growth itself (Campbell and Holt, 2001). This finding is most likely because this synthesis can only produce insufficient amounts of proteins to supply a large amount of cytoskeleton or membrane components to the growing axon.

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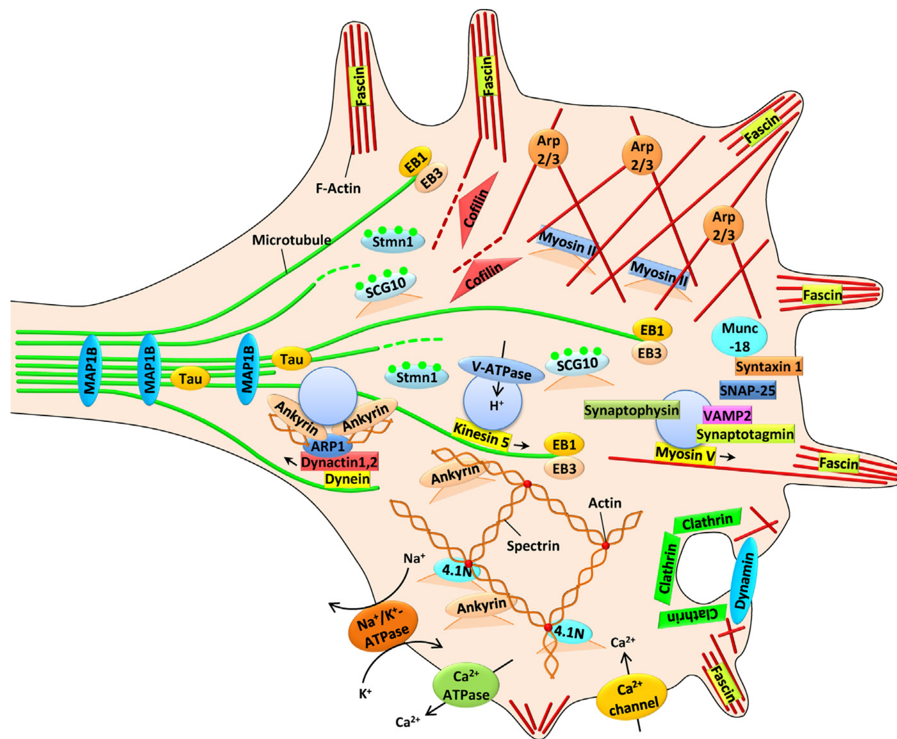

Fig. 3. A scheme of the mammalian CNS growth cone whose functions are attributed to the major proteins identified by proteomics. Red, F-actin; green, microtubules. See Section 5.

Holt and her colleagues reported a list of mRNAs localized in the growth cones of *Xenopus* by directly cutting the growth cones from cultured *Xenopus* neurons, collecting the mRNAs, and sequencing the cDNAs corresponding to the mRNAs produced by RT-PCR. Their list contained more than 1000 coding mRNAs (Zivraj et al., 2010). Evidently, only some mRNAs are utilized for local protein synthesis, and the sufficient synthesized proteins should most likely be restricted in number. Studies to determine which proteins are highly synthesized should performed using proteomic analysis again.

5.6. Ion channels and transporters

Because the growth cone is primarily involved in neuronal wiring in an activity-independent manner, the ionic channels for electrical activity are few (such as Na^+ channels). Among the transporters in the growth cone identified by proteomics, the most abundant transporter is Na^+/K^+-ATPase, and the amounts of these transporters are as large as NCAM or L1 (Nozumi et al., 2009). This result is surprising because this molecule is not clearly shown in relation to nerve growth with sufficient evidence, including the growth cones of model organisms. One possibility is that this ATPase is electrogenic and is involved in the membrane potentials of the growth cone because Na^+ channels, which primarily primary function in the mature neuron, are not expressed in the early stage of the GCM. The second most abundant transporter is Ca^{2+}-ATPase localized in the plasma membrane (PMCA1 and PMCA2); these molecules are suggested to have a central role in cation movements across the growth cone membrane (Boczek et al., 2012). Additionally, V-ATPase, which plays important roles in endosomal/lysosomal/vesicular acidification (Igarashi et al., 1997), is also localized in the GCM fraction, suggesting that the acidification process may be important.

Recently, TRPV channels, which are sensitive to temperature and mechanical stimuli, have been featured as new types of ion channels. Among these channels, TRPV2 has been shown to have activity role in inducing axon growth (Shibasaki et al., 2010).

5.7. Metabolic enzymes

Undoubtedly, metabolic features in the growth cone should contribute its functions; however, the metabolomic approach, which comprehensively and quantitatively identifies the metabolic substances (metabolites) of the growth cone, has not yet been achieved. Thus, the proteomic features of the metabolic enzymes certainly help us understand this point, although it may be too early to allow definite conclusions.

Some features of the enzymes are described below.

5.7.1. Fatty acid synthesis

Fatty acid synthase (FAS), which is an enzyme that synthesizes palmitate (16:0), is one of the most abundant metabolic enzymes in GCPs. Even compared with synaptosomes, this enzyme is considerably enriched in GCPs. This enzyme synthesizes C16 or shorter FAs in the cytosol, and longer FAs are synthesized by four membrane-bound enzymes after palmitate is transported to the ER (Reizman, 2007). One of these enzymes, 2,3-enoyl reductase (TER), is an enzyme required for the last of four steps, and this enzyme is identical to GPSN2, which is known as the abundant protein in the mammalian brain (Moon and Horton, 2003). We also identified this molecule as one of the nGAPs (M. Nozumi and M.I., in preparation). Interestingly, these enzymes are involved in synthesizing very long chain fatty acids (VLCFAs), such as eicosapentaenic acid (EPA) and docoxahexaenic acid (DHA), which are well-known enriched fatty acids in the mammalian CNS. Recently, a mutation in the coding region of human TER was found in a genetic change in hereditary mental retardation (Çalışkan et al., 2011; Abe et al., 2013).

Fatty acid binding proteins (FABPs) are involved in the cellular transport of fatty acids to intracellular organelles or to the nucleus and are thought to contribute to the transcriptional regulation of

lipophilic molecules (Liu et al., 2010). One brain-type FABP is called FABP7, which is primarily expressed in astrocytes of the mature brain. However, judging from the developmental aspects, this protein is transiently present in neurons (Liu et al., 2010). This protein is one of the nGAPs; thus, the local synthesis of fatty acids and their transport are suggested to be one of the novel mechanisms for nerve growth (Lu et al., 2008, 2011).

Collectively, the local synthesis and transport of fatty acids may greatly contribute to regulating nerve growth, and further intensive studies to evaluate this novel hypothesis are required.

5.7.2. Other enzymes

Except for the ubiquitous distribution of glycolytic enzymes, brain-type creatine (phosphor) kinase (CK) is notably present at a high level (Béard and Braissant, 2010). The knockout mice of the enzyme do not have developmental abnormalities (Zandt et al., 2004); however, its significance in the growth cone function should be studied.

5.8. Protein degradation systems

Proteasome components and ubiquitination-related enzymes were identified in GCPs. These results indicate that ubiquitin-dependent proteolysis is a major system in the growth cone. However, E3 ubiquitin ligases, which have many members, were present in low abundance. These molecules, i.e., Nedd4 and Pam/highwire (PHR/Phr1), have already been reported to have roles in axon growth, even in model animals. For example, PHR is thought to regulate microtubule stability, and Nedd4 is reported to regulate PTEN and Robo signaling (Myat et al., 2002; Keleman et al., 2005). However, E3 ligase has many substrates, and identifying important mammalian targets for axon growth or guidance should be required (Hendricks and Jesuthasan, 2009).

5.9. Molecular chaperones

Some reports have indicated that chaperones are involved in regulating axonal growth or guidance. For example, *Xenopus* FKBP52 may regulate an ion channel required for netrin-dependent guidance (Shim et al., 2009).

5.10. Unknown proteins

Using proteomics, we have identified many types of proteins whose functions are unclear or completely unknown, particularly regarding growth cone functions. For example, one of these proteins is the glycoprotein M6a (GPM6a). This protein, which is a DM20/PLP family member (relatives to myelin proteolipid protein) with four transmembrane regions, is highly concentrated in the neurons after the neuronal stem cells differentiate (Möbius et al., 2008). Overexpression of this protein induces filopodia formation; however, its physiological importance was not demonstrated (Sato et al., 2011). Using interactomic methods, we recently found that GPM6a is involved in polarity determination (A. Honda and M.I., in preparation).

6. Concluding remarks

I have summarized the putative functions of identified proteins using molecular maps of the mammalian CNS growth cone (Fig. 3). Using the pathway analysis, these profiles should depict the novel putative signaling pathways that regulate growth cone behavior. The verification of these pathways seems easier than using pharmacological methods alone. However, clearly, conventional inactivation methods, such as knockout mouse techniques, take a long time to perform and to understand the roles of the many proteins revealed by proteomics. Together with the development of imaging using superresolution microscopy, which has recently been developed with high resolution to determine the exact localization of proteins, these large pieces of the molecular information derived from our proteomics will greatly contribute the above purpose. We have begun such approaches as an ongoing project. Another important approach is bioinformatics. The interactome database and pathway analysis will provide us with the missing link for the components present in small amounts and that have not been convincingly identified using usual proteomic methods, including the pathway analysis, which combines the protein–protein interactions and signaling pathways. In addition, phosphoproteomic analysis is a more powerful method for finding the signaling mechanisms among the proteins identified by proteomics.

Another important approach is the simulation model, which represents the growth cone functions based on the molecular concentrations, distributions, and interactions of these proteins. This approach should be indispensable if a large number of proteins have clearly been demonstrated to be involved in the regulation of the growth cone (Kobayashi et al., 2010). For example, Goodhill and his colleagues reported a model used to determine the direction of the growth cone advance by second messenger concentrations (Forbes et al., 2012).

Soon, combining these approaches should convincingly provide us with the true views of the complicated molecular signaling pathways regulating the growth cone behavior in the mammalian CNS and enable us to provide an overview regarding the molecular mechanisms of growth cone behavior.

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