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REVIEW ARTICLE

Involvement of semaphorins and their receptors in neurological diseases

Noriko Takegahara[1,2] and Atsushi Kumanogoh[1,2]

[1]Department of Immunopathology, Research Institute for Microbial Diseases, and [2]World Premier International Research Center, Immunology Frontier Research Center, Osaka University, Osaka, Japan

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Correspondence
Atsushi Kumanogoh, Department of Immunopathology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan.
Tel: +81-6-6879-8333
Fax: +81-6-6879-8332
Email: kumanogo@ragtime.biken.osaka-u.ac.jp

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Abstract

The immune and nervous systems have various common features in their functional characteristics. Both have an intricate network of synaptic connections and an exquisite communication system that enables intercellular signal transduction. Semaphorins were originally identified as guidance factors for developing neuronal axons. However, accumulating evidence indicates that several semaphorins called "immune semaphorins" are crucial for various phases of immune responses, from the initiation to the termination of inflammatory processes. Furthermore, it is becoming clear that immune semaphorins contribute to pathological immune responses in the central nervous system. Here, we review the present knowledge of the function of semphorins and their receptors in the immune system, and their involvement in the pathogenesis of neurological diseases. (Clin. Exp. Neuroimmunol. doi: 10.1111/j.1759-1961.2009.00004.x, January 2010)

Introduction

There are many links between the immune and nervous systems. Both are highly networked systems that interact with each other using shared molecules such as chemical mediators and cytokines.[1] The immune response is composed of a series of cell–cell contacts, including interactions between T cells and antigen-presenting cells (APC) such as B-cells, macrophages and dendritic cells (DC). Such cell–cell contact elicits the activation of immune responses, characterized by clonal expansion and development of effector functions of T cells in which the T cell receptor (TCR) forms a close contact with the cognate antigen peptide-major histocompatibility complex on the cell surface of APC. This structure is termed the "immunological synapse", which is similar to "neurological synapse".

Semaphorins, named for their analogy to the system of signaling flags used in maritime communications, are chemorepulsive factors required for guiding neuronal axons to appropriate targets. Since semaphorins were first described in the early 1990s, more than 20 types of these proteins have been identified (Fig. 1).[2–4] Although they were originally identified as axonal guidance factors,[4] semaphorins are currently known to have diverse and important functions in other physiological processes[5] including heart morphogenesis,[6] vascular growth,[7,8] tumor progression[9–11] and immune cell regulation.[12–14]

Semaphorins are secreted and membrane-associated proteins characterized by a conserved amino-terminal "Sema" domain. The semaphorins range in size from 400 to 1000 amino acid residues depending on additional C-terminal sequence motifs such as an immunoglobulin domain, thrombospondin domain or glycosylphosphatidylinositol (GPI) linkage site. On the basis of structural elements and amino acid sequence similarities, the family has been divided into eight subclasses. Invertebrate semaphorins are grouped into classes I and II, whereas classes III–VII are expressed in vertebrates. In addition, some DNA viruses encode functional semaphorin proteins. Semaphorins in classes I and IV–VII are membrane associated, whereas those in classes II, III and the viral semaphorins are secreted. Two groups of proteins, plexins and neuropilins, have been identified as the primary receptors for semaphorins.[15,16] Most

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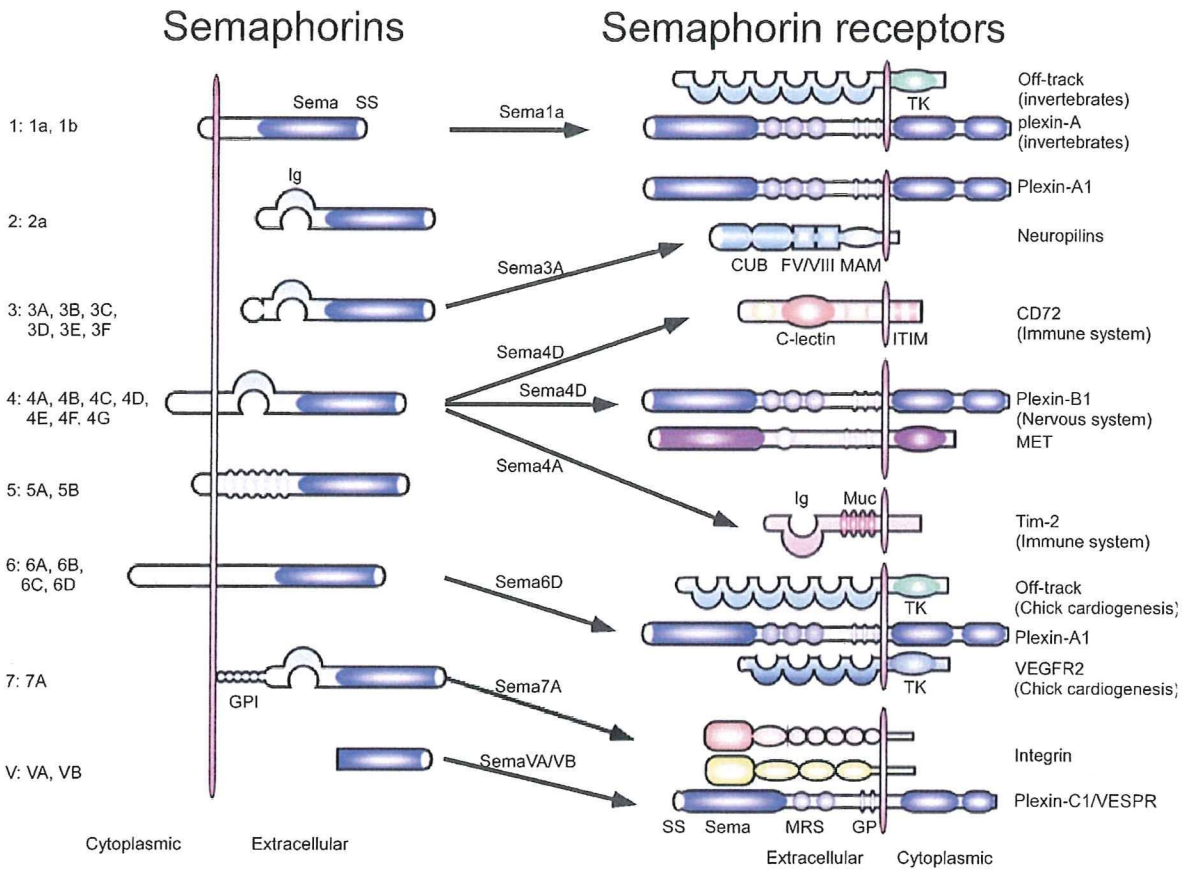

Figure 1 Semaphorins and their receptors. Class I and II semaphorins are found in invertebrates and class III–VII are vertebrate semaphorins. Classes II and III, and viral semaphorins are secreted, whereas class IV–VI are transmembrane. Class VII represents glycosylphosphatidylinositol-anchored proteins. The major semaphorin receptors are plexin family proteins. Plexins are categorized into four groups (A, B, C and D) and also carry sema domains. Another group of semaphorin receptors are neuropilins (neuropilin-1 and neuropilin-2), which form receptor complexes with plexin-A family members and exclusively bind to class III semaphorins. Plexins associate with several transmembrane molecules such as Met, Off-track and VEGFR2. In addition, in the immune system some semaphorins use non-plexin receptors such as CD72 and TIM-2.

membrane-bound semaphorins bind plexins directly, but class III semaphorins require neuropilins as obligate co-receptors.[17] However, recent reports have suggested that receptor usage by semaphorins is more complex than previously thought. For example, Sema3E signals independently of neuropilin through plexin-D1,[18] and Sema7A uses integrin receptors to exert its function in both the nervous and immune systems.[19,20] Some plexins further associate with various co-receptors to exert the diverse functions of semaphorins.[10,21,22] Additionally, in the immune system two molecules unrelated to plexins and neuropilins, CD72[23] and T cell immunoglobulin and mucin domain protein-2 (TIM-2),[24] functionally interact with Sema4D and Sema4A, respectively. The functions of semaphorins and their receptors have been shown by using gene-targeted mice (Table 1).

Understanding of the immunoregulatory functions of the semaphorin family has advanced considerably over the past several years. These semaphorins are currently called "immune semaphorins" (Fig. 2).[12,14] Furthermore, cumulative evidence shows that immune semaphorins are pathologically involved in various immune compromised diseases, including immune mediated-neurodegenerative disorders. In the present review, we will describe the most recent knowledge of their pathological involvement in neurological disorders.

Immune semaphorins

Sema4D: a semaphorin involved in B cell/DC activation

Sema4D, also known as CD100, is the first semaphorin protein of which immunoregulatory func-

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Table 1 Phenotypes of semaphorin/semaphorin receptor/coreceptor-knockout mice

Knockout molecules	Phenotypes in non immune systems	Phenotypes in the immune system
Sema3A	Abnormalities in peripheral nerve projection	Enhanced T cell proliferation
Sema4A	Abnormalities in retinal formation	Impaired T cell priming Impaired helper T cell differentiation
Sema4D	Enhancement of motor activity	Impaired B cell activation and humoral immune responses Impaired T cell priming
Sema7A	Abnormalities in lateral olfactory tract formation	Impaired macrophage activation and inflammatory responses
Neuropilin-1	Abnormalities in the trajectory of efferent fibers of the peripheral nerve projection	Hyperproliferation of T cells (in Neuropilin-1sema-mutant mice)
Plexin-A1	Defective in the organization of cutaneous afferents	Impaired DC activation and T cell priming Impaired osteoclast development and develop osteopetrosis
Plexin-A4	Defective in the trajectory and projection of peripheral sensory axons	Hyperproliferation of T cells and enhanced T cell priming
Plexin-B1	Major defects has not been detected	Not reported
TIM-2	Not reported	Enhanced basal proliferation of T cells Dysregulated Th2 responses
CD72	Not reported	Enhanced B cell activation
Integrin $\alpha 1\beta 1$	Develop hypocellular dermis	Inhibition of effector phase inflammatory responses
TREM-2	Not reported	Enhanced cytokine production by stimulation with TLR ligands
DAP12	Developmental arrest of oligodendrocytes and develop hypomyelinosis	Impaired T cell priming Impaired osteoclast development and develop osteopetrosis

tions were identified. In the immune system, the expression of Sema4D is detectable in resting T cells.[25,26] The basal expression of Sema4D in B cells and DC is very low, but it is considerably upregulated after cellular activation.[23]

Sema4D promotes B cell activation in the context of proliferation and antibody production.[23] Regarding the receptors for Sema4D, plexin-B1[16,23,27,28] and CD72[23] have been identified in the nervous and immune systems, respectively. CD72 contains two immunoreceptor tyrosine-based inhibitory motifs (ITIM) in its cytoplasmic domain.[29,30] CD72 is known to function as a negative regulator of B cells through the recruitment of a tyrosine phosphatase SHP-1 to its phosphorylated ITIM.[31,32] Ligation of Sema4D to CD72 induces the dissociation of SHP-1 from CD72, resulting in the activation of B cells.[23] Sema4D-deficient mice display impaired antibody production.[33] implicating Sema4D in B cell activation.

In addition to the involvement of Sema4D in B-cell responses, it also plays a role in T cell responses through the activation of DC.[34] Sema4D expressed on T cells interacts with its cognate receptor on DC to promote the activation and maturation of DC, resulting in enhanced T cell activation. In fact, Sema4D-deficient mice display impaired generation of antigen-specific T cells.

Although Sema4D is a transmembrane protein, the extracellular region is proteolytically cleaved from the surface of activated lymphocytes by a metalloprotease-dependent process.[35] Sema4D is also cleaved from the surface of platelets by the metalloprotease ADAM17.[36] The elevation of the levels of soluble Sema4D protein is detectable in culture supernatants of activated lymphocytes and in the sera of either immunized or autoimmune mice.[35]

Sema4A: a semaphorin involved in both T cell activation and differentiation

Sema4A is another class IV semaphorin. Sema4A is expressed in a broad range of adult tissues, including the brain, lung and spleen. In the immune system, Sema4A is constitutively expressed on DC.[24] The expression of Sema4A is also detectable in activated T cells and T helper type 1 (Th1)-polarized cells.[37] DC-derived Sema4A and T cell-derived Sema4A play different roles during the course of T cell-mediated immunity. DC-derived Sema4A is crucial for antigen-specific T cell priming,[24] whereas T cell-derived Sema4A is involved in helper T cell differentiation.[37] Indeed, the critical involvement of Sema4A in the differentiation of helper T cells has been shown by the phenotypes of Sema4A-deficient mice. Sema4A-deficient mice show impaired responses to heat-killed *Propionibacterium acnes*, a Th1-inducing agent. Conversely, Sema4A-deficient mice show enhanced T helper type 2 (Th2) responses against infection of *Nippostrongylus brasiliensis*, a Th2-inducing intestinal

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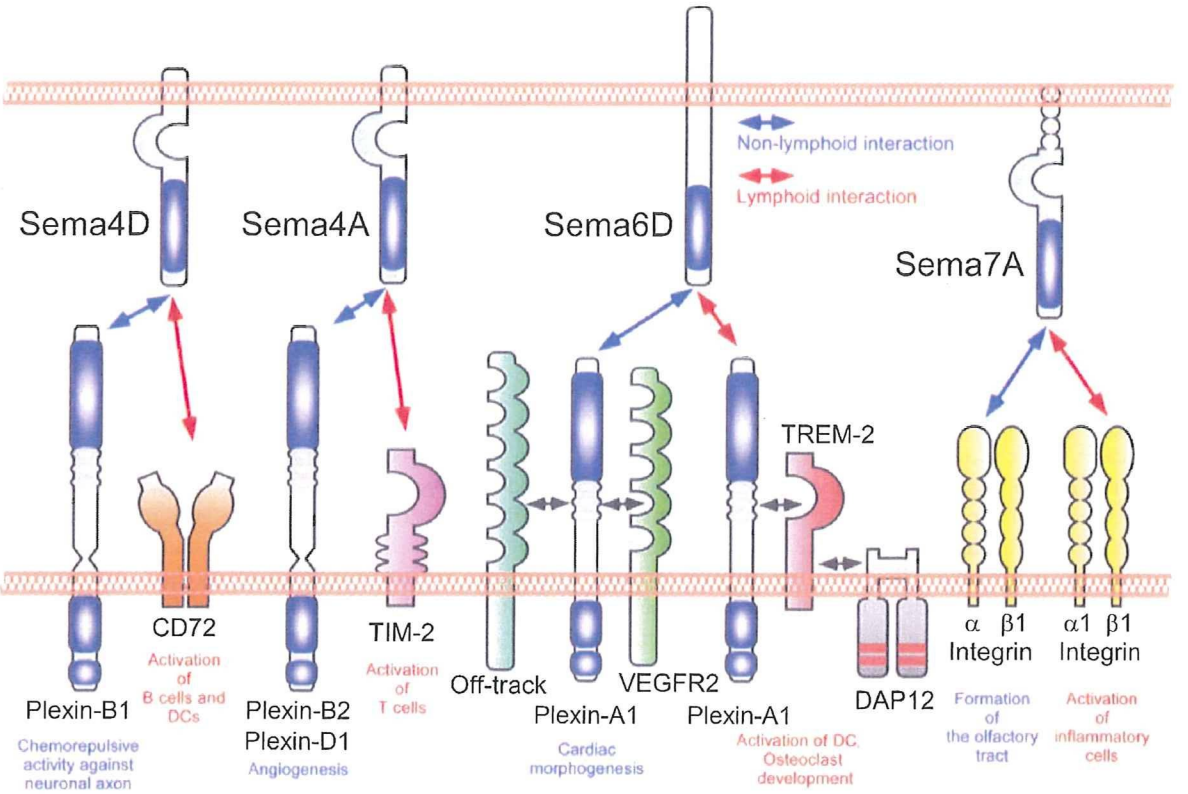

Figure 2 Representative immune semaphorins and their receptors in lymphoid and non-lymphoid cells. Although Sema4D binds to plexin-B1 in the brain and transduces chemorepulsive signals, plexin-B1 couples with Met in epithelial cells and induces Sema4D-mediated cell outgrowth. In the immune system, Sema4D uses CD72 as a functional receptor in B cells and dendritic cells (DC), and enhances the activation of B cells and DC. Sema4A binds TIM-2 and is involved in T cell activation and differentiation in the immune system. In the non-immune system, however, Sema4A recognizes plexin-B proteins and plexin-D1. Sema6D exerts different biological activities through plexin-A1, depending on its co-receptors. During chick embryogenesis, plexin-A1 differentially associates with Off-track and VEGFR2, and these receptor complexes have distinct functions in heart development. In the immune system, plexin-A1 forms a receptor complex with TREM-2 and DAP12 and, after Sema6D binds, this complex transduces signals that stimulate DC and osteoclasts. Sema7A uses β1 integrin as receptors in both the nervous and immune systems. In the immune system, Sema7A expressed on activated T cells stimulates macrophages through α1β1 integrin to promote inflammatory responses.

nematode.[37] Furthermore, Sema4A-deficient mice on a Th2-prone BALB/c background spontaneously develop atopic dermatitis (AD) (T.M. unpublished data), supporting the notion that Sema4A is involved in the regulation of Th1/Th2 development.

In the immune system, TIM-2-expression is induced on activated T cells.[24] Several lines of evidence support that TIM-2 serves as a functional receptor for Sema4A. The expression of TIM-2 is preferentially upregulated on T cells during Th2 differentiation. Administration of recombinant TIM-2 protein suppresses the development of experimental autoimmune encephalomyelitis (EAE) in SJL is suitable mice immunized with proteolipid protein-derived peptide by inhibiting the generation of Th1 cells.[38] Furthermore, TIM-2-deficient mice show exacerbated lung inflammation accompanied by dysregulated Th2 responses.[39] Taken together, it is tempting to speculate that Sema4A-TIM-2 interactions negatively regulate Th2 responses. However, there are some phenotypic differences between Sema4A-deficient and TIM-2-deficient mice. For example, T cells from TIM-2-deficient mice but not from Sema4A-deficient mice show enhanced basal proliferation. The observation raises the possibility that Sema4A and/or TIM-2 have other binding partners. Indeed, T cells express members of plexin-B proteins and plexin-D1, both of which have Sema4A-binding activities.[7]

Sema6D and plexin-A1: an interaction involved in T cell-dendritic cell interface and osteoclastogenesis

Plexins function as semaphorin receptors during the development of the nervous and cardiovascular

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systems. Plexin-A1 functions have been extensively investigated in both the nervous and cardiovascular systems. Class III semaphorins bind a receptor complex formed by plexin-A1 and neuropilin-1. Additionally, plexin-A1 serves as a direct binding receptor for class VI semaphorins, Sema6C, and Sema6D.[21,40]

In the immune system, plexin-A1 is specifically expressed by DC. The function of plexin-A1 in DC is shown using an RNA interference system and analysis of plexin-A1 knockout mice. "Knockdown" of plexin-A1 in DC by short hairpin RNA impairs their ability to activate T cells *in vitro* and *in vivo*.[41] In addition, plexin-A1-deficient DC poorly stimulate antigen-specific T cells.[22] Furthermore, plexin-A1-deficient mice show impaired T cell-priming. These observations indicate that plexin-A1-expression in DC is required for the initial activation and efficient generation of antigen-specific T cells.[22] Additionally, plexin-A1 is involved in osteoclast differentiation, that is, plexin-A1-deficient mice develop osteopetrosis as a result of decreased bone reabsorption by defective osteoclastogenesis.[22]

Regarding the ligand of plexin-A1 in the immune system, Sema6D was identified as a putative ligand for plexin-A1 in the regulation of DC function.[22] The expression of Sema6D mRNA is detectable in T cells, B cells and natural killer (NK) cells. Recombinant Sema6D protein binds to and activates DC, and these activities are profoundly attenuated in plexin-A1-deficient DC.[22] These observations suggest that Sema6D-expression on T cells is involved in DC-activation. The expression of Sema6D is also observed in osteoclasts.[22] Recombinant Sema6D protein enhances *in vitro* osteoclastogenesis, suggesting that Sema6D-plexin-A1 might function in osteoclastogenesis in an osteoclast-autonomous manner. Plexin-A1 forms a receptor complex with the receptor-triggering receptor expressed on myeloid cell-2 (TREM-2) and the adaptor molecule DAP12 in DC and osteoclasts.[22] DAP12-deficient mice show impaired T cell responses and develop osteopetrosis,[42,43] and genetic mutations of human DAP12 or TREM-2 result in a bone fracture syndrome called Nasu-Hakola disease, supporting the idea that plexin-A1 physiologically associates with TREM-2/DAP12 complex.

Sema7A: a semaphorin involved in inflammatory responses

Sema7A, also known as CD108, is a membrane-associated GPI-linked protein. Sema7A transcripts are detectable in the embryonic nervous system and in adult tissues, including the brain, spinal cord, lung and secondary lymphoid organs.[44,45] In the nervous system, Sema7A has been shown to promote olfactory bulb axon outgrowth and is required for the proper formation of the lateral olfactory tract during embryonic development.[19] Plexin-C1 was initially identified as a receptor for Sema7A.[46] However, Sema7A contains an arginine–glycine–aspartate sequence that is a well conserved integrin-binding motif in its Sema domain, and it exerts its function through β1 integrin, not through plexin-C1.[19]

In the immune system, the expression of Sema7A is induced on activated T-cells,[45] and it is involved in T cell-mediated inflammatory immune responses.[20] Recombinant Sema7A protein stimulates monocytes/macrophages through α1β1 integrin, also known as very late antigen-1, inducing the production of proinflammatory cytokines.[20] Consistently, Sema7A-deficient mice are resistant to the development of inflammation, including hapten-induced contact hypersensitivity and experimental autoimmune EAE.[20] These observations indicate that interactions between Sema7A and α1β1 integrin is crucial for T cell-mediated macrophage activation at sites of inflammation.[20] Plexin-C1 is also expressed in macrophages. However, stimulation with recombinant Sema7A protein induces normal production of proinflammatory cytokines by plexin-C1-deficient macrophages (unpublished data). Therefore, at least for the T cell–macrophage interactions, α1β1 integrin but not plexin-C1 seems to be the predominant receptor for Sema7A. Furthermore, integrin-mediated signaling is a common mechanism for Sema7A-functions in both the nervous and immune systems.

Sema3A and plexin-A4: a semaphorin and its receptor required for negative regulation of T cell responses

Sema3A is the first semaphorin identified in vertebrates. Its function as an axon repellent has been well established. Sema3A directly binds to neuropilin-1, which induces activation of plexin-A proteins and the transduction of axon repulsive signals. Several lines of evidence suggest that Sema3A also functions in the immune system. The expression of Sema3A is detected in activated DC, T cells and some tumor cells. Sema3A inhibits spontaneous monocyte migration *in vitro*. In addition, Sema3A suppresses T cell proliferation by inhibiting actin cytoskeletal reorganization and downregulating MAPK signaling.[36,47] Furthermore, Sema3A-deficient T cells exhibit enhanced *in vitro* proliferative responses to anti-CD3 antibodies.[48] These observations suggest that Sema3A serves as a negative regulator of T cells.

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Similar to other plexin-A proteins, plexin-A4 forms a receptor complex with neuropilin-1 to transduce class III semaphorin-mediated signaling or directly binds to Sema6A.[49] In the immune system, the expression of plexin-A4 is observed in various cells including T cells, DC and macrophages, but not in B and NK cells.[48] Plexin-A4-deficient T cells exhibit hyperproliferation and enhanced TCR signals on anti-CD3 stimulation.[48] Furthermore, plexin-A4-deficient mice show enhanced T cell priming and exacerbated T cell-mediated immune responses such as EAE.[48] Therefore, plexin-A4 might interact with Sema3A in the immune system and this interaction might negatively regulate T cell responses. However, it remains unclear how plexin-A4 negatively regulates T cells and whether other semaphorins are relevant to plexin-A4-mediated immune responses.

Neuropilin-1: a marker for regulatory T cells

As described above, neuropilin-1 was originally identified as a cell surface glycoprotein that acts as a class III semaphorin receptor. Neuropilin-1 is also known as human DC-specific antigen (blood DC antigen)-4, a specific plasmacytoid DC marker in humans, and was assigned CD304. In the immune system, the expression of neuropilin-1 was observed in DC and T cells.[50] Neuropilin-1 has been thought to be involved in the initiation of primary immune responses through a homophilic interaction at the contact sites between T cells and DC.[50] In addition, neuropilin-1 has been identified as a specific marker for $CD4^+CD25^+$ regulatory T (Treg) cells.[51] Indeed, neuropilin-1 is part of the group of forkhead box P3 (Foxp3)-inducible genes, including CD25, glucocorticoid-induced tumor necrosis factor receptor-related protein and cytotoxic T-lymphocyte antigen-4 (CTLA-4).[51]

More recently, one report suggested that neuropilin-1 in Treg cells contributes to the long contact between Treg cells and DC compared with the shorter contact between naive T cells and DC.[52] Treg cells made stable contact with DC that precedes the contact of naive T cells with DC, and this might lead to the inhibition of T-cell activation in the steady state. The finding that Treg cells are endowed with the ability to have long interactions with DC by neuropilin-1 supports the idea that neuropilin-1 might contribute to physical interaction between T cells and DC. However, it remains to be elucidated whether the long contact is mediated by a homophilic interaction such as semaphorins and neuropilin-1 or neuropilin-1-associating molecules such as plexins.

Semaphorins and neurological disorders

A large body of evidence has shown that the immune system is sometimes deleterious for the survival of neurons and the maintenance of central nervous system (CNS) integrity.[53] Once rapid and extensive neuronal death occurs as a result of infections, trauma, stroke or ischemic injury, CNS integrity is compromised and activated microglia or lymphocytes, especially T cells, inevitably come into contact with neurons. This accelerates degeneration of neurons concomitantly with increased expression of proinflammatory molecules. Neuroinflammation has been associated with a wide range of neurological disorders, including Alzheimer's disease, Parkinson's disease and multiple sclerosis (MS).[54,55] In accordance with their expression in the brain and their roles in immune responses, it is emerging that immune semaphorins and their receptors are crucially involved in the protection and/or progression of inflamed disorders in the CNS (Table 2). In this section, we will discuss the roles of immune semaphorins and their receptors in neurological disorders.

Multiple sclerosis, EAE and semaphorins

Multiple sclerosis is an immune-mediated chronic disease characterized by disseminated foci of inflammatory demyelination affecting the CNS. Although the pathogenesis of MS has not yet been elucidated, the conventional hypothesis assumes a T cell-mediated autoimmune reaction against unknown myelin antigens because there is an accumulation of activated T cells in nervous tissues, a hallmark of autoimmune diseases of the CNS. Myelin oligodendrocyte glycoprotein (MOG)-induced EAE is an established animal model of MS. EAE is, in part, mediated by neuroantigen-reactive T cells, especially $CD4^+$ T cells. In this model, inflammatory demyelination and axon loss as a result of a concerted T cell response are observed. Cumulative evidence shows that some immune semaphorins and their receptors play a role in the development of EAE through regulation of antigen-specific T cell immunity.

Sema4D and EAE

As described earlier, Sema4D expressed on T cells is crucially involved in the initial activation of T cells through maturation of DC.[34] When Sema4D-deficient mice are immunized with a MOG-peptide in Freund's complete adjuvant (CFA), they show attenuated development of EAE. $CD4^+$ T cells from the draining lymph nodes of immunized Sema4D-

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Table 2 Immune semaphorins, their receptors and neurological diseases

Semaphorins/receptors	Expression in the immune system	Binding partner	Immunological activities	Related neurological diseases
Sema3A	ND	Plexin A proteins	Inhibition of monocyte migration Inhibition of T cell activation	Alzheimer's disease Atopic dermatitis
Sema4A	Dendritic cells Activated-T cells Th1 cells	Plexin B proteins Plexin-D1 TIM-2	T cell activation Promotion of Th1-differentiation	EAE Atopic dermatitis
Sema4D	T cells Activated-B cells Dendritic cells	Plexin-B1 CD72	B-cell activation DC-activation	EAE HAM
Sema5A	ND (Oligodendrocytes)	ND	ND	Parkinson's disease
Sema6D	T cells B cells NK cells	Plexin-A1	DC-activation	
Sema7A	Acticated-T cells	Plexin-C1 Integrin $\alpha 1\beta 1$	Monocyte/macrophage-activation	Contact hypersensitivity EAE
Neuropilin-1	T cells Treg cells	Class III semaphorins		Alzheimer's disease
Plexin-A1	Dendritic cells (Osteoclasts)	Class VI semaphorins	DC-activation	EAE
Plexin-A4	T cells Dendritic cells Macrophages	Class VI semaphorins	Inhibition of T-cell activation	EAE
Plexin-B1		Class IV semaphorins		
TIM-2	Acticated-T cells Th2 cells	Sema4A	T-activation	EAE Airway atopy
CD72	B cells Dendritic cells	Sema4D	B cell activation DC-activation	
Integrin $\alpha 1\beta 1$	Monocytes Macrophages	Sema7A	Monocyte/macrophage-activation	EAE

deficient mice exhibit impaired antigen-specific T cell responses, particularly the generation of cytokine-producing effector cells, after *in vitro* restimulation with antigen. These observations indicate the involvement of Sema4D in the pathogenesis of EAE during the interaction between T cells and DC.

Sema4A and EAE

Sema4A expressed on DC is involved in the initial activation of T cells.[24] Development of MOG-induced EAE in wild-type mice can be suppressed by intravenous injection of anti-Sema4A monoclonal antibody concurrently with MOG immunization.[24] Infiltration of mononuclear inflammatory cells into the spinal cord is diminished in anti-Sema4A antibody-treated mice, in which MOG-peptide-specific responses of $CD4^+$ T cells isolated from the draining lymph nodes are greatly decreased. Thus, blocking Sema4A with anti-Sema4A monoclonal antibody inhibits generation of MOG-peptide-specific $CD4^+$ T cells, leading to attenuated development of EAE.[24] T helper type 17 (Th17) cells, $CD4^+$ T cells that secret IL-17, play a critical role in inflammatory pathology in autoimmune diseases, including MS. Because the function of Sema4A is shown to be important for helper T cell differentiation,[37] it is plausible that Sema4A is involved in both the priming and effector phases of EAE through regulation of Th17 cell development even though the relevance of Sema4A in the development of Th17 cells has not been clarified. Ongoing studies will clarify the involvement of semaphorins in the development of Th17 cells and the pathogenesis of EAE.

Sema7A and EAE

Sema7A is involved in T cell-mediated inflammation through the activation of peripheral macrophages.[20] When Sema7A-deficient mice are immunized with MOG-peptide in CFA, the T cells are primed normally and generate MOG-peptide-specific $CD4^+$ T cells. However, these mice are resistant to EAE development. $CD4^+$ T cells from MOG-immunized Sema7A-deficient mice fail to induce EAE when they are transferred into naïve wild-type mice. In addition, MOG-peptide-primed $CD4^+$ T cells from wild-type mice fail to induce EAE on transfer into

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α1 integrin-deficient mice. Furthermore, Sema7A on antigen-primed effector T cells plays a role in the induction of inflammation in EAE through interaction with α1β1 integrin, and contribute to the exacerbation of EAE.[20] These findings show the pathological involvement of Sema7A in the effector phase of EAE, of which functional sites seem to be different from those of class IV semaphorins.

Plexin-A1 and EAE

Plexin-A1 expressed on DC is involved in the generation of antigen-specific T cells.[22] Immunization of plexin-A1-deficient mice with MOG-peptide in CFA results in impaired development of EAE in accordance with impairment of MOG-peptide-specific $CD4^+$ T cell responses.[22] Consistent with the finding that DAP12 associates with plexin-A1, DAP12-deficient mice exhibit attenuated development of MOG-induced EAE and impaired generation of MOG-specific T cells.[42,56]

Plexin-A4 and EAE

Plexin-A4 is also involved in the pathology of EAE. As described above, plexin-A4 negatively regulates T cell immunity.[48] In accordance with *in vitro* hyper responses of plexin-A4-deficient $CD4^+$ T cells, plexin-A4-deficient mice exhibit enhanced generation of antigen-specific-T cells and exacerbated EAE when they are immunized with MOG-peptide in CFA.[48] On transfer to naïve wild-type mice, $CD4^+$ T cells from MOG-immunized plexin-A4-deficient mice can induce a more severe EAE than cells from wild-type mice.[48] Collectively, these observations indicate that plexin-A4 is involved in the pathology of EAE, although its contribution is precisely the opposite to that of plexin-A1. It is interesting to determine why these closely related molecules, plexin-A1 and plexin-A4, play opposite functions in T cell immunity; plexin-A1 expressing DC are required for the induction of effective T cell immunity, whereas plexin-A4 expression by T cells is required for the regulation of excess T cell responses. Further studies are required to clarify the detailed mechanism underlying the opposing functions of these two molecules.

Parkinson's disease and semaphorins

Parkinson's disease is a neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra and the presence of neuronal intracellular Lewy bodies. The clinical features of Parkinson's disease are resting tremor, rigidity, bradykinesia and postural instability. Recently, a link between Sema5A and Parkinson's disease has been reported. Sema5A is a member of class V semaphorin proteins. The extracellular domain of Sema5A contains seven thrombospondin type-1 repeats following the Sema domain.[57] In the nervous system, Sema5A is expressed by oligodendrocytes and inhibits axonal growth,[58] and has been shown to be essential for the development of the extra-embryonic tissues and the cardiovascular system.[59,60] A high resolution whole genome association study showed that a single-nucleotide polymorphism within SEMA5A (rs7702187) is associated with Parkinson's disease.[61] In addition, polymerase chain reaction-restriction fragment length polymorphism analysis showed an association of Sema5A haplotypes with Parkinson's disease risk in the Chinese Han population, although the analysis showed no significant association of variant genotypes of Sema5A with the risk of Parkinson's diseases in the population.[62,63] Not only genetic factors but also environmental factors have been suggested to be risk factors for Parkinson's disease. Further studies are needed to understand the exact mechanism of the pathogenesis of Parkinson's disease.

Alzheimer's disease and semaphorins

The neurodegeneration of selectively vulnerable hippocampal CA1 and subicular pyramidal neurons is a hallmark of the earliest pathogenesis of Alzheimer's disease. Neurodegenerative changes in hippocampal CA1 and subiculum are observed during the incipient phases of Alzheimer's disease, and progress during further pathogenesis of the disease. The relationship between Alzheimer's disease and semaphorins was first reported in 1999, in which the altered expression pattern of class IV semaphorins was reported in the brains of patients with Alzheimer's disease.[64] Several lines of evidence suggest the association of semaphorin-plexin signaling with Alzheimer's disease. Progressive accumulation of Sema3A proteins was detected in hippocampal CA1 and subicular neurons in Alzheimer's disease.[65] In addition, phosphorylated microtubule associated protein 1B, collapsin-response mediator protein-2 (CRMP-2), plexin-A1 and plexin-A2 were also detected in the hippocampus of patients with Alzheimer's disease.[65] CRMP-2 has been identified as an intracellular signaling molecule in the Sema3A signaling pathway. Phosphorylated CRMP-2 was detectable in neurofibrillary tangles in Alzheimer's disease,[66–68] and stimulation with Sema3A enhanced the levels of the phosophorylated form of CRMP-2.[69] Collectively,

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these observations suggest that aberrant Sema3A signaling might contribute to the degeneration of neurons in the CA1 field of the hippocampus of Alzheimer's disease patients.

Amyotrophic lateral sclerosis and semaphorins

Amyotrophic lateral sclerosis (ALS) is a devastating disease characterized by progressive neurodegeneration of motor neurons in the brain and spinal cord.[70] The loss of motor neurons results in paralysis of voluntary muscles and eventual death by respiratory failure within 1–5 years of the disease. A direct relationship between Sema3A and ALS was recently reported. Transgenic mice that express a dominant gain-of-function mutant of superoxide dismutase-1 ($SOD1^{G93A}$), a mouse model for ALS, displayed a marked increase of Sema3A expression in terminal Schwann cells (TSC).[71] This increase was limited to TSC on a specific subset of muscle fibers known as fast-fatigable or type IIb and IIx muscle fibers. This subtype of muscle fiber has been characterized by its inability of stimulate nerve sprouting after injury and is the first muscle subtype that is lost in ALS. Although an increased expression of Sema3A was also found in adult TSC,[71] developmental expression changes might be involved in inappropriately repelling motor axons away from the neuromuscular junctions and predispose patients to ALS.

Human T lymphotropic virus type 1 associated myelopathy and semaphorins

Myelopathy associated with human T lymphotropic virus type 1 infection (tropical spastic paraparesis/HTLV-1-associated myelopathy) is a neuroinflammatory disease characterized by inflammation with white matter damage and axonal degeneration in the brain and spinal cord. Both infiltrating T cells and inflammatory mediators are suspected to participate in the pathogenic mechanisms of HAM. Soluble forms of Sema4D have been reported to be increased in the cerebrospinal fluid and spinal cords of patients with HAM.[72] Activated T cell-derived Sema4D induces apoptotic cell death of multipotent neural progenitors and immature oligodendrocytes, both of which are required for re-myelination and neuronal integrity.[72] These observations suggest that Sema4D might function in the deleterious cross talk between T cells and neuronal cells during neuroinflammation, thus playing a role in demyelination or inhibiting re-myelination in neuroinflammatory diseases such as HAM.

Neuroinflammation, other neurological disorders and semaphorins

Sema3A, neuropilin-1 and plexin-A1 play roles in T cell responses and T cell-mediated immunity.[22,48,50,52] Recent studies also showed the involvement of these molecules in the protection of neurons through interactions between neurons and microglia. In the rat CNS, stress signals induced the upregulation of Sema3A in neurons while activation of microglia induced upregulation of plexin-A1 and neuropilin-1.[73] Culture with recombinant Sema3A induced apoptosis-mediated cell death of microglia and a similar result was obtained on activated microglia on coculture with stressed neurons which produce Sema3A.[73] It has been shown that the interaction with apoptotic neurons induces microglial cells to release neuroprotective agents, such as anti-inflammatory cytokines and growth factors, while inhibiting the production of nitric oxide and proinflammatory cytokines.[74] Thus, these observations indicate that Sema3A expressed by stressed neurons might serve to protect them from further damage by microglia through the promotion of microglial cell death.

Interestingly, contributions of Sema3A on the treatment of AD have been reported. In AD patients, C-fibers in the epidermis increase and sprout, inducing hypersensitivity, which is thought to aggravate the disease. Administration of recombinant Sema3A to the skin lesions of NC/Nga mice, an animal model of AD, resulted in the improvement of skin lesions and attenuation of the scratching behavior in NC/Nga mice.[75] Histological examination showed a decrease in epidermal thickness and the density of invasive nerve fibers in the epidermis.[75] In addition, infiltration of immune cells, such as mast cells and $CD4^+$ T cells, also decreased.[75] These observations suggest an alleviative effect of Sema3A on AD. Because the interruption of the itch–scratch cycle likely contributes to the improvement of the atopic-dermatitis lesions, Sema3A will become a good pharmacological target for treatment of AD patients.

Regarding co-receptors of plexin-A1, dysfunction of TREM-2 or DAP12 is known to be the cause of Nasu-Hakola disease.[43,76,77] Nasu-Hakola disease is characterized by a combination of bone fractures and psychotic symptoms similar to schizophrenia, rapidly progressing to presenile dementia. A recent study has identified a role for TREM-2 expression by microglia in Nasu-Hakola disease.[78] In the nervous system, the expression of TREM-2 was detectable in microglia. Knockdown of TREM-2 in microglia by

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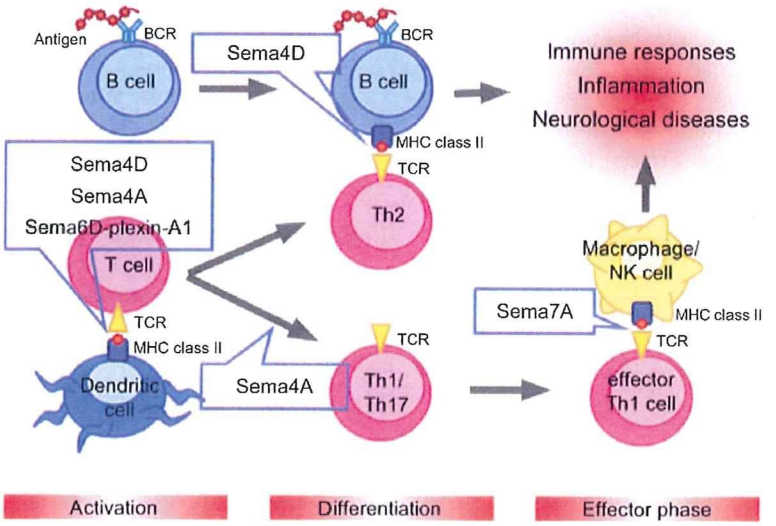

Figure 3 Involvement of immune semaphorins in various phases of immune responses. Several semaphorins and their receptors have distinct biological activities in various phases of immune responses. In the initial phase of T cell immune responses, Sema4D and Sema6D expressed by T cells activate dendritic cells (DC) through their receptors, CD72 and plexin-A1, respectively. Sema4A expressed on DC is also involved in T cell priming through its binding partner, TIM-2, of which expression is induced on activated T cells. After the activation of T cells, the expression of Sema4A is specifically induced on Th1 cells, and the specific expression of Sema4A is required for efficient Th1 differentiation. In contrast, Sema4D is upregulated on activated T cells and promotes humoral immune responses through the activation of B cells. Differentiated effector T cells express Sema7A on their cell surface, and interactions between T cell expressing Sema7A and macrophage expressing $\alpha 1\beta 1$ integrin induce macrophage activation, resulting in the promotion of inflammatory responses. BCR, B cell receptor; MHC, major histocompatibility complex; TCR, T cell receptor.

siRNA showed impaired phagocytosis of apoptotic neurons and increased gene transcription of tumor necrosis factor and nitric oxide synthase-2, whereas overexpression of TREM-2 in microglia increased phagocytosis and decreased microglial proinflammatory responses.[78] Thus, TREM-2-deficiency resulted in impaired clearance of apoptotic neurons and inflammation that might be responsible for the brain degeneration observed in patients with Nasu-Hakola disease. Because of the association of TREM-2 with plexin-A1, it is conceivable that plexin-A1 (or other plexin-A proteins) might be involved in the function of microglia through regulation of their phagocytic activities.

Concluding remarks

So far, it has been speculated that there is communication between neurons and immune cells. However, the molecules that mediate the communication have remained undetermined. Immune semaphorins, newcomers to the growing panoply of immunoregulatory proteins, are good candidates. Recently, it has emerged that CNS neurons actively participate in immune regulation by controlling infiltrated T cells through direct or indirect interactions. Further studies will clarify the functions of immune semaphorins in the regulation of immune responses and neuroinflammation in the CNS (Fig. 3).

Beyond the basic implications, studies of immune semaphorins have also provided valuable insights into therapeutic strategies for compromised immune disorders including neuroinflammatory diseases. So far, various immune therapies are shown to be potentially beneficial for the treatment of MS and Alzheimer's disease. However, concomitant side-effects, such as unexpected autoimmune reactions induced by these therapies, and the potential harmful effects of over-activated microglia on neurons might be problematic. In this context, it is important to minimize the underlying damaging potential of an inflammatory response while keeping it active enough to promote CNS repair. Advances in understanding the functions of immune semaphorins and their receptors at the interface between the nervous and immune systems will shed light on this problem and help us to better combat these diseases.

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Disclosure

The authors declare that they have no competing financial interests.

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Sema4A induces cell morphological changes through B-type plexin-mediated signaling

KAZUNORI YUKAWA[1], TETSUJI TANAKA[2], KENJI YOSHIDA[1], NORIKO TAKEUCHI[1], TAKUJI ITO[1], HYOTA TAKAMATSU[3], HITOSHI KIKUTANI[4] and ATSUSHI KUMANOGOH[3]

[1]Department of Physiology, Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tempaku-ku, Nagoya 468-8503; [2]Department of Obstetrics and Gynecology, Wakayama Medical University, 811-1 Kimiidera, Wakayama 641-8509; Department of [3]Immunopathology; [4]Molecular Immunology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan

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Abstract. Semaphorins are a family of secreted and membrane-bound proteins known as axonal pathfinders. Sema4A, a member of class 4 semaphorins, induces growth cone collapse of hippocampal neurons. The binding of Sema4A to growth cones indicates the presence of receptors transmitting signals through the intracellular effectors to induce growth cone collapse in hippocampal neurons. Transfection experiments of the candidate receptor genes into COS-7 cells demonstrated that Sema4A binds to axonal guidance receptors Plexin-B1, -B2 and -B3. To identify the functional Sema4A receptor and the signal transduction machinery, COS-7 cell contraction assay was performed, in which intracellular signal transmission induced by Sema4A triggered cell contraction. Expression vectors encoding plexins and Rnd1, a Rho family GTPase, were transfected into COS-7 cells, and a proportion of contracted cells among the transfectants was determined after incubation with Sema4A. The results demonstrated that the combination of Rnd1 and Plexin-B1, -B2 or -B3 induced significant cell contraction, indicating that B-type plexins transmit an intracellular signal of Sema4A through Rnd1. To further study the mechanism of B-type plexin-mediated signaling in Sema4A-induced growth cone collapse, mouse hippocampal neurons transfected with a control or expression plasmid encoding a constitutively active mutant of R-Ras (R-RasQL) were stimulated with Sema4A, followed by the assessment of growth cone collapse. Expression of R-RasQL significantly blocked Sema4A-induced growth cone collapse in the hippocampal neurons compared with the control plasmid. Sema4A thus induces growth cone collapse through the down-regulation of R-Ras activity in mouse hippocampal neurons.

Correspondence to: Dr Kazunori Yukawa, Department of Physiology, Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tempaku-ku, Nagoya 468-8503, Japan
E-mail: kyukawa@ccmfs.meijo-u.ac.jp

Key words: semaphorin, axon guidance, growth cone collapse, small guanosine triphosphatase, cell contraction, R-Ras

Introduction

Semaphorins compose a large family of phylogenetically conserved soluble and transmembrane molecules, which are further divided into eight classes (1). Semaphorins were originally identified as repulsive axonal guidance cues which induce growth cone collapse in developing neurons (2-4). Later studies have revealed their widespread roles in a variety of developmental and pathological conditions (5,6). Sema4D, a member of class 4 semaphorins, has facilitated our understanding of how semaphorins regulate axonal repulsion through cytoskeletal changes (7-10). Sema4D, also known as CD100, induces repulsive changes including growth cone collapse in cultured hippocampal neurons and retinal ganglion cells (7). For the repulsion, Sema4D binds to Plexin-B1, a member of the plexins that are the predominant family of semaphorin receptors (7). Both Sema4D and Plexin-B1 contain a distinctively conserved ~400 amino acid Sema domain that features a seven-blade ß-propeller fold in their extracellular domain (1,3) and they interact with each other through their respective Sema domain (5). In the intracellular region at the C terminus, Plexin-B1 has two GTPase activating protein (GAP) domains separated by a GTPase binding domain and a PDZ-binding site (5,7,8). Binding of Sema4D to Plexin-B1 induces clustering of Plexin-B1 leading to the activation of the GAP domains, which is facilitated by an active GTPase Rnd1-dependent relief of the GAP domain interaction (8). The GAP activity of Plexin-B1 promotes the conversion of GTP-bound (active) R-Ras to GDP-bound (inactive) R-Ras, resulting in the downregulation of R-Ras activity. This in turn downregulates integrin-based cell adhesion to the extracellular matrix, thus leading to growth cone collapse in cultured hippocampal neurons (8). Plexin-B1 also allows intracellular RhoA-specific guanine nucleotide exchange factors (GEFs), PDZ-RhoGEF and leukemia-associated RhoGEF (LARG), to bind to the PDZ-binding motif at the

C terminus of Plexin-B1 (7). Sema4D binding to Plexin-B1 stimulates the GEF activities of PDZ-RhoGEF and LARG, promoting the conversion of the GDP-bound form of RhoA, a member of Rho GTPases crucial for the regulation of actin and microtubule dynamics (7), to the GTP-bound form. The increase in the GTP-bound forms of RhoA enhances actomyosin contractility through Rho kinase activation and myosin light chain phosphorylation, thereby leading to growth cone collapse of hippocampal neurons (5,7). Both the R-Ras GAP activity and PDZ-RhoGEF-mediated RhoA activation by plexin-B1 are necessary for the Sema4D-induced growth cone collapse in cultured hippocampal neurons (7,8).

In mouse, the expression of Sema4A, another class 4 semaphorin originally identified as semB, gradually increases during embryonic development (11) and becomes prominent in the adult brain, spleen, lung, kidney, testis and mammary gland (12). Sema4A expressed in dendritic cells and B-cells enhances the activation of T-cells and the generation of antigen-specific T-cells by virtue of the interaction with the Sema4A receptor, Tim-2, a member of the T-cell immunoglobulin domain and mucin domain (Tim) proteins expressed on activated T-cells (12). Our previous study showed that recombinant Sema4A induces growth cone collapse in mouse hippocampal neurons (13). The binding of Sema4A to the growth cones indicated the presence of a specific receptor transmitting Sema4A signal via the intracellular effectors to induce growth cone collapse in the neurons (13). In the current study, to identify the functional Sema4A receptor and the signal transduction machinery, we first performed COS-7 cell contraction assay, in which Sema4A-induced intracellular signaling triggered cell contraction (14). The assay was performed to examine whether one of the B-type plexins (Plexin-B1, -B2 or -B3) and Rnd1, a small GTP-binding protein of the Rho family, constituted a signal transduction machinery of Sema4A. Using primary hippocampal neurons, we also investigated whether the intrinsic guanosine triphosphatase (GTPase) activity of R-Ras, a member of the Ras superfamily of small GTP-binding proteins, is regulated in Sema4A-induced growth cone collapse in mouse hippocampal neurons.

Materials and methods

Expression plasmids and antibodies. Expression plasmids encoding hemagglutinin (HA)- and GFP-tagged Rnd1, Myc-tagged Plexin-B1, -C1 and HA-tagged R-Ras-QL (Q87L) were generously provided by Dr M. Negishi, Kyoto University, Japan (8). Expression plasmids encoding VSV-G-tagged Plexin-B2, -B3 and -D1 were generous gifts from Dr L. Tamagnone, University of Torino, Italy. The following antibodies were used in this study: mouse monoclonal antibody against Myc (Santa Cruz Biotechnology, Santa Cruz, CA), a rat monoclonal antibody against HA (Roche, Mannheim, Germany), a mouse monoclonal antibody against VSV-G (Roche), a mouse monoclonal antibody against GFP conjugated to Alexa 594 (Molecular Probes, Eugene, OR), and secondary antibodies conjugated to Alexa 594 (Molecular Probes).

Production of Sema4A-Fc. Recombinant soluble mouse Sema4A protein comprising the putative extracellular region fused to the human immunoglobulin-γ1 (IgG1) Fc fragment (Sema4A-Fc) was prepared as previously described (12).

Cell culture and transfection. COS-7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 4 mM glutamine, 100 units/ml penicillin, and 0.2 mg/ml streptomycin at 37°C in a humidified 5% CO_2/95% air incubator. COS-7 cells were transiently transfected with one or a combination of expression plasmids in lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Primary hippocampal neurons were isolated from embryos from pregnant mice at embryonic day 17 and cultured on glass coverslips in 24-well plates as described previously (15,16) except for the use of the Sumilon Nerve-Cell Culture System (Sumitomo Bakelite, Tokyo, Japan). Primary hippocampal neurons on glass coverslips were transfected with Lipofectamine 2000 containing expression plasmids after 12 h in culture.

Growth cone collapse. Hippocampal neurons cultured from E17 mouse embryos for 1.5 days were treated with Sema4A-Fc (10 μg/ml) or its vehicle, for 1 h at 37°C. Neurons were fixed with 4% paraformaldehyde and processed for immunocytochemistry. FITC-labeled Phalloidin (Sigma, St. Louis, MO, USA) was used to detect actin filaments in growth cones. Neurons were scored for the presence of growth cones. Growth cones with round-tipped or pencil-like appearance represented the collapsed form (17,18). Data were expressed as a percentage of collapsed growth cones out of the total number of growth cones, and the mean values were calculated from four separate experiments.

COS-7 cell binding and contraction assay. COS-7 cells ($1x10^4$ cells) were seeded onto round 10-mm glass coverslips coated with lysine/laminin and transfected with expression plasmids encoding mouse and human Plexin-B1, -B2, -B3, -C1, -D1, Tim-2 and/or Rnd1. After transfection (24 h), COS-7 cells were incubated with recombinant Sema4A fused with heat stable alkaline phosphatase (Sema4A-AP) for 90 min at room temperature. Cells were postfixed for 30 sec in a buffer containing 60% acetone, 3% formaldehyde, and 20 mM HEPES (pH 7.5), followed by heating at 65°C for 20 min to inactivate endogenous alkaline phosphatase. The binding of Sema4A to COS-7 cells was visualized by reaction with NBT/BCIP (19). For COS-7 cell contraction assay, COS-7 cells transiently transfected with individual plasmids were treated 24 h after the transfection with Sema4A-Fc (10 μg/ml) or its vehicle for 5 min at 37°C. The size of transfected cells was visualized using a fluorescence microscope and their images were captured with a digital camera (HV-C20S; Nikon, Tokyo, Japan).

Statistics. All data represent means ±SEM. Statistical significance was determined by analysis of variance, and $p<0.05$ was considered as a minimal level of significance.

Results

Sema4A binds to B-type plexins. To examine which guidance receptors Sema4A binds to, binding assays with COS-7 cells expressing candidate receptors were performed using

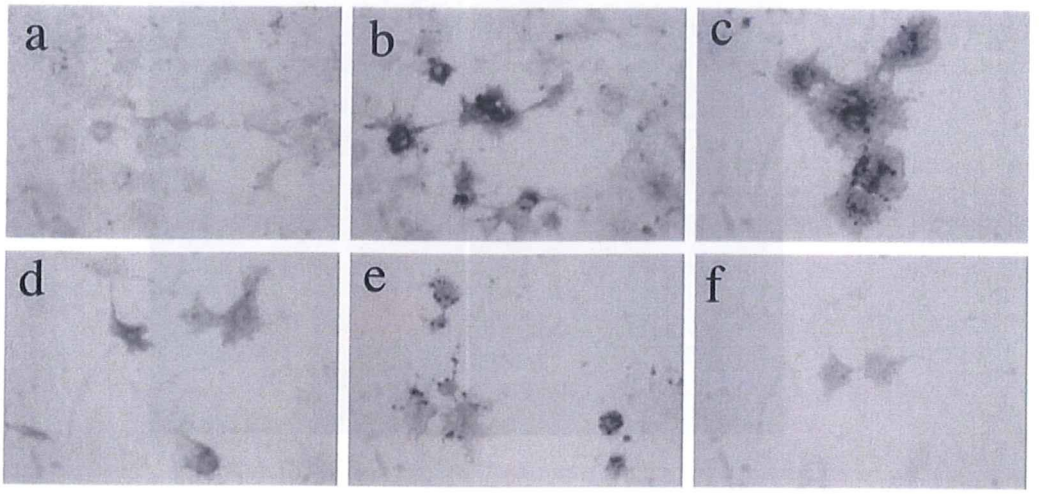

Figure 1. Sema4A binds to Plexin-B1, -B2 and -B3. Sema4A binds to COS-7 cells expressing mouse Plexin-B1 (b), human Plexin-B1 (c), human Plexin-B2 (d), and human Plexin-B3 (e) on lysine/laminin-coated coverslips. COS-7 cells were initially transfected with expression plasmids encoding EGFP (a), mouse Plexin-B1 (b), human Plexin-B1 (c), Plexin-B2 (d), Plexin-B3 (e) and Plexin-C1 (f), and then the binding ability of Sema4A to each plexin was examined with Sema4A-AP.

recombinant Sema4A fused to heat stable alkaline phosphatase. As shown in Fig. 1, the assay revealed the binding of Sema4A to COS-7 cells expressing one of the B-type plexins (Plexin-B1, -B2, or -B3). Recombinant Sema4A did not exhibit significant binding to COS-7 cells expressing either the control vector or Plexin-C1 (Fig. 1). Thus, the binding assays showed that Sema4A interacts specifically with B-type plexins.

Sema4A uses B-type plexins and Rnd family GTPase, Rnd1, to induce cell contraction. To identify functional Sema4A receptor and the signal transduction machinery, COS-7 cell contraction assay was performed, in which Sema4A-induced intracellular signal transmission leads to cell contraction (14). Sema4A did not induce any morphological changes in COS-7 cells expressing Plexin-B1 alone (Fig. 2A). However, COS-7 cells expressing both Plexin-B1 and Rnd1 showed significant contraction of the cell body (Fig. 2A). Quantitative analysis of the COS-7 cell contraction was performed to obtain the percentage of cells showing contraction out of the total number of the transfected cells. The results showed that cells coexpressing Plexin-B1 and Rnd1 exhibited significantly higher collapses than cells transfected with the control plasmid (Fig. 2B). The assay also revealed that Sema4A induced significant contraction of COS-7 cells expressing both Plexin-B2 and Rnd1 (Fig. 2B) as well as Plexin-B3 and Rnd1 (Fig. 2B).

Involvement of R-Ras in the Sema4A-induced growth cone collapse. Downregulation of R-Ras activity by a Plexin-B1-Rnd1 complex is integral to the growth cone collapse in hippocampal neurons induced by Sema4D, another class 4 semaphorin (8). To examine whether the downregulation of R-Ras activity is also involved in Sema4A-induced growth cone collapse in hippocampal neurons, mouse hippocampal neurons expressing a constitutively active mutant of R-Ras (R-RasQL) were treated with Sema4A, and growth cone morphology was analyzed by immunofluorescence methods.

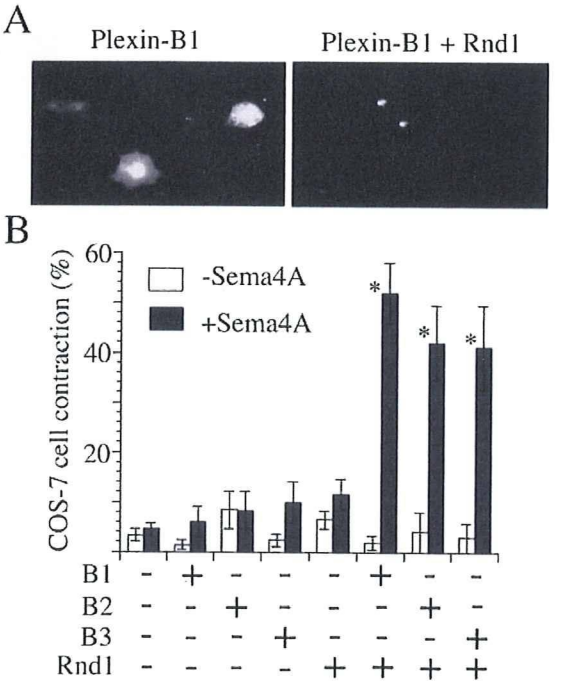

Figure 2. Sema4A-induced COS-7 cell contraction is mediated by B-type plexin and Rnd1. (A) COS-7 cells were transfected with the indicated plasmids and incubated with Sema4A for 5 min 24 h after transfection. The transfected cells were visualized by the fluorescence from the antibodies against GFP, Myc, and HA. Sema4A did not induce contraction of COS-7 cells overexpressing Plexin-B1 alone, whereas Sema4A induced significant contraction of COS-7 cells coexpressing Rnd1 and one of the B-type plexins (Plexin-B1, -B2, or -B3). The scale bar represents 10 μm. (B) Quantitative analysis of COS-7 cell contraction mediated by Plexin-B1, -B2, -B3, or Rnd1 in response to Sema4A binding. COS-7 cells were transfected with an expression vector encoding HA tag, GFP, Plexin-B1, -B2, -B3, or Rnd1. After transfection (24 h), cells were incubated with Sema4A for 5 min. Double positive cells with both HA (or GFP) and Myc-staining were counted and COS-7 cell contraction was expressed as a percentage of contracted cells out of the total transfected cells. About 50 cells were assessed in one experiment, and the data represent the mean ±SEM values of three independent experiments. B1, Plexin-B1; B2, Plexin-B2; B3, Plexin-B3.

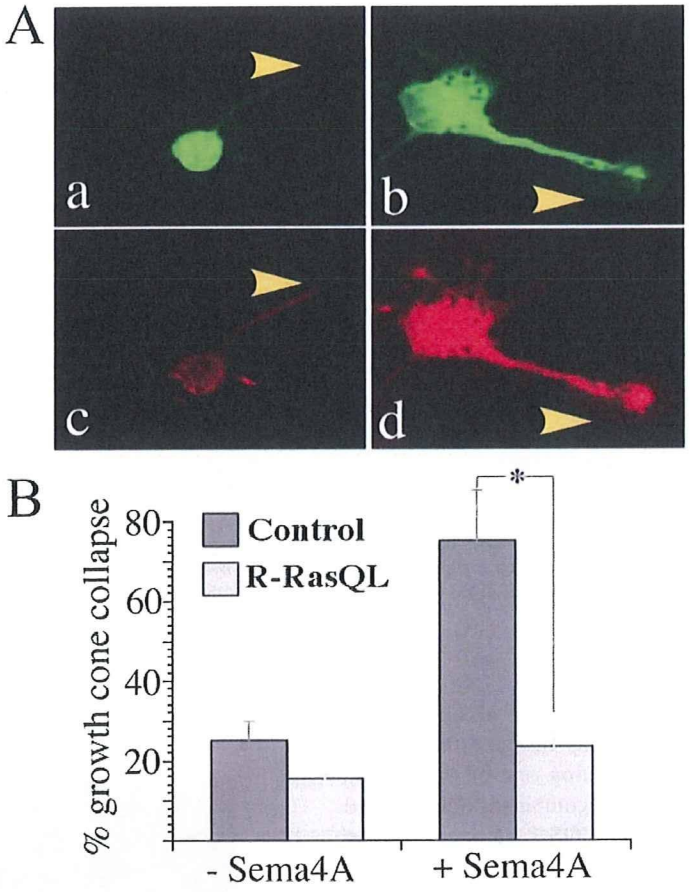

Figure 3. Downregulation of R-Ras activity is required for the Sema4A-induced growth cone collapse in mouse hippocampal neurons. (A) Mouse hippocampal neurons transfected with a control or expression plasmid encoding a constitutively active mutant of R-Ras (R-RasQL) were stimulated with Sema4A. Growth cones of the transfectants were shown by the immunofluorescence from the antibody against HA tag. The presence of filopodia and lamellipodia visualized by the binding of Alexa 594-conjugated phalloidin to filamentous actin (F-actin) defined a growth cone. Sema4A induced growth cone collapse in the primary hippocampal neurons expressing the control vector (arrowhead in a and c). In contrast, Sema4A did not induce growth cone collapse in the hippocampal neurons expressing R-RasQL (arrowhead in b and d). (B) Growth cone collapse was scored as a percentage of the transfected neurons with collapsed growth cones out of the total number of transfected cells. Hippocampal neurons with the control vector exhibited significantly higher growth cone collapses in response to Sema4A (+ Sema4A), as compared with hippocampal neurons expressing the control vector or R-RasQL cultured without Sema4A (- Sema4A). Expression of R-RasQL, but not the control plasmid, significantly blocked Sema4A-induced growth cone collapse in mouse hippocampal neurons.

Sema4A induced growth cone collapse in primary hippocampal neurons expressing the control vector (Fig. 3A-a and -c). In contrast, hippocampal neurons expressing R-RasQL did not exhibit growth cone collapse in response to Sema4A (Fig. 3A-b and -d). Quantitative assay measuring the percentage of the transfected neurons with collapsed growth cones out of the total number of the transfected cells clearly showed that the overexpression of R-RasQL significantly blocked the Sema4A-induced growth cone collapse in the primary hippocampal neurons compared with the overexpression of the control plasmid (Fig. 3B).

Discussion

Our molecular and cellular analyses have revealed that Sema4A induces B-type plexin-mediated collapse of COS-7 cells through Rnd1 and growth cone collapse of mouse hippocampal neurons through the downregulation of R-Ras activity. Our findings suggest the crucial involvements of Sema4A in the neurodevelopmental processes via the activation of B-type plexin-mediated signaling.

The binding of Sema4A to B-type plexins in this study is consistent with the recent finding that showed the binding of ^{125}I-labeled Sema4A to the membranes of cells expressing one of B-type plexins or Plexin-D1 (20). In the vascular system, Sema4A binds to Plexin-D1 expressed on the endothelial cells and mediates antiangiogenic activity (20). In the immune system, Sema4A binds to Tim-2 on T-cells, leading to the activation of their proliferation and differentiation (12). Induction of Plexin-D1 expression allows Sema4A to reduce cell adhesion to fibronectin and collagen type 4, but not to poly-D-lysine and laminin (20). Another study reported that Sema4C, another member of class 4 semaphorins, weakly enhanced adhesion of granule cell populations to laminin, but not to poly-L-lysine or fibronectin (21). In our experimental conditions using lysine/laminin coated glass coverslips as matrices, the binding of Sema4A was observed only to B-type plexins but not to either Plexin-C1 or -D1. This may indicate

that both the binding of semaphorins to plexin receptors and the physiological effects of semaphorin-plexin interaction vary depending on the context generated by the combination of various cell types and extracellular matrices.

In our COS-7 cell contraction assay, Sema4A induced significant contraction of COS-7 cells coexpressing Rnd1 and one of the B-type plexins. Plexin-B1 functions as a molecule with R-Ras GAP activity (8), and other B-type plexins are also believed to have R-Ras GAP activities (5). It is necessary for Rnd1 to bind to plexin-B proteins to induce their R-Ras GAP activity inside cells (8,22). Our experimental results showed that, upon binding to plexin-B proteins expressed on the COS-7 cell membranes, Sema4A signaled cells to induce morphological changes, that is, cell contraction, through Rnd1.

In our experiments to explore if Sema4A exhibits any physiological roles in the directional guidance of axonal elongation during brain development, overexpression of the constitutively active mutant of R-Ras in cultured hippocampal neurons significantly blocked growth cone collapse induced by Sema4A. Sema4D promotes growth cone collapse in hippocampal neurons by binding to plexin-B1. Upon Sema4D binding, Plexin-B1 through the interaction with Rnd1 enhances the GAP activity toward R-Ras, leading to the downregulation of R-Ras/integrin pathway and thus to the decrease in the adhesion of growth cones to extracellular matrices (8,9,22). It is therefore possible that Sema4A also promotes growth cone collapse of cultured hippocampal neurons by stimulating R-Ras GAP activities through B-type plexins. Recent studies investigating the roles of semaphorins revealed not only the crucial involvements of each semaphorin in axon guidance but also in the ligand-receptor interactions between semaphorins and plexins essential for neural development (21-25). Plexin-B2 is not only a crucial component of neural tube development but also a key molecule that directs proliferation and migration of granule cell precursors in the developing dentate gyrus, olfactory bulb, and cerebellum (21). The expression pattern, high affinity and specific nature of Sema4C-Plexin-B2 binding and their functional studies consistently propose Sema4C as a true ligand for Plexin-B2 (21). Plexin-B3 has been reported to interact *in vitro* with a member of class 5 semaphorins, Sema5A (23). The analysis of Plexin-B1 knockout mice has shown that Plexin-B1 is integral for gonadotropin hormone-releasing hormone-1 (GnRH-1) cells to migrate from olfactory placode to hypothalamic areas (24). Sema4D promotes directional migration in immortalized GnRH-1 neurons by coupling Plexin-B1 with activation of the Met tyrosine kinase (24). However, because of the failure to detect migratory defects of GnRH-1 neurons in Sema4D-deficient mice, the reality of the semaphorin ligand controlling the migration of GnRH-1 cells remains controversial (24). This raises the possibility that many semaphorins including Sema4D and Sema4A function in concert to regulate the migration of GnRH-1 cells *in vivo*. Analysis of Sema4A-deficient mice revealed the non-redundant roles of Sema4A in the developmental path of retinal photoreceptor cells, although the receptors involved have not yet been clarified (25). For these reasons, Sema4A may play a crucial role in the development of the neural system through the interaction with B-type plexins in concert with other semaphorin members.

In summary, our study showed that the binding of Sema4A to Plexin-B1, -B2 or -B3 *in vitro* leads to the B-type plexin-mediated contraction of COS-7 cells through Rnd1. Our study further demonstrated that Sema4A promotes growth cone collapses of mouse hippocampal neurons through the down-regulation of R-Ras activity. Our results therefore suggest the crucial involvement of Sema4A in the growth cone guidance through the activation of B-type plexin-mediated signaling in developing neurons.

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ORIGINAL ARTICLE

Sema4D/CD100 Deficiency Leads to Superior Performance in Mouse Motor Behavior

Kazunori Yukawa, Tetsuji Tanaka, Noriko Takeuchi, Hiroyuki Iso, Li Li, Akira Kohsaka, Hidefumi Waki, Masayasu Miyajima, Masanobu Maeda, Hitoshi Kikutani, Atsushi Kumanogoh

ABSTRACT: ***Background:*** Sema4D/CD100 is a type of class 4 semaphorin, exhibiting crucial roles in growth cone guidance in developing neurons. Sema4D is widely expressed throughout the central nervous system in embryonic mouse brain, and is selectively localized to oligodendrocytes and myelin in the postnatal brain. However, direct evidence of the actual involvement of Sema4D in the neuronal network development crucial for neurobehavioral performance is still lacking. The present study therefore examined whether Sema4D deficiency leads to abnormal behavioral development. ***Methods:*** Both wild-type and Sema4D-deficient mice were subjected to behavioral analyses including open-field, adhesive tape removal, rotarod tests and a water maze task. ***Results:*** Open-field tests revealed increased locomotor activity in Sema4D-deficient mice with less percentage of time spent in the center of the field. In both the adhesive tape removal and rotarod tests, which examine motor coordination and balance, Sema4D-deficient mice showed significantly superior performance, suggesting facilitated motor behavior. Both Sema4D-deficient and wild-type mice successfully learnt the water maze task, locating a hidden escape platform, and also showed precise memory for the platform position in probe tests. However, the swimming speed of Sema4D-deficient mice was significantly faster than that of wild-type mice, providing further evidence of their accelerated motor behavior. ***Conclusion:*** Our mouse behavioral analyses revealed enhanced motor activity in Sema4D-deficient mice, suggesting the crucial involvement of Sema4D in the neurodevelopmental processes of the central structures mediating motor behavior in mice.

RÉSUMÉ: Performance supérieure du comportement moteur chez la souris ayant un déficit en Sema4D/CD100. ***Contexte :*** Sema4D/CD100 est un type de sémaphorine de classe 4 qui joue des rôles cruciaux dans le guidage des cônes de croissance dans les neurones en développement. Sema4D est largement exprimé dans tout le système nerveux central de l'embryon chez la souris et il est localisé sélectivement aux oligodendrocytes et à la myéline du cerveau après la naissance. Cependant, il n'y a pas de preuve directe de l'implication de Sema4D dans le développement du réseau neuronal qui est crucial pour le fonctionnement neurocomportemental. Dans cette étude, nous avons examiné si un déficit en Sema4D entraîne un développement comportemental anormal. ***Méthodes :*** Des souris de phénotype sauvage et des souris déficientes en Sema4D ont été soumises à des analyses comportementales, dont le test du champ ouvert, de l'adhesive tape removal, du rotarod et du labyrinthe aqueux. ***Résultats :*** Les tests de champ ouvert ont montré que les souris ayant un déficit en Sema4D avaient une activité locomotrice accrue et passaient un pourcentage moindre du temps dans le centre du champ. Dans les tests de l'adhesive tape removal et du rotarod, qui évaluent la coordination motrice et l'équilibre, les souris ayant un déficit en Sema4D ont présenté une performance significativement supérieure, ce qui est en faveur d'un comportement moteur facilité. Les souris ayant un déficit en Sema4D et les souris de type sauvage ont appris avec succès la tâche du labyrinthe aqueux qui consistait à localiser une plate-forme de sauvetage dissimulée et ont également fait preuve d'une mémoire précise de la position de la plate-forme lorsque le test était répété après avoir retiré la plate-forme. De plus, la vitesse de nage des souris ayant un déficit en Sema4D était significativement supérieure à celle des souris de type sauvage, ce qui est en faveur d'un comportement moteur accéléré. ***Conclusion :*** Nos analyses comportementales chez la souris ont démontré que les souris ayant un déficit en Sema4D ont une activité motrice accrue, ce qui est en faveur du rôle crucial que joue Sema4D dans le processus de développement nerveux des structures centrales, soit un rôle facilitant sur le comportement moteur chez la souris.

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From the Departments of Physiology, Faculty of Pharmacy (KY, NT), Meijo University, Tempaku-ku, Nagoya; Departments of Obstetrics & Gynecology (TT, LL) Physiology (AK, HW, MMa), and Animal Facility (MMi), Wakayama Medical University, Wakayama; Department of Behavioral Science (HI), Hyogo College of Medicine, Nishinomiya; Department of Molecular Immunology (HK) and Immunopathology (AK), Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan.

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Correspondence to: Kazunori Yukawa, Department of Physiology, Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tempaku-ku, Nagoya 468-8503, Japan.