Binding of laminin-1 to monosialoganglioside GM1 in lipid rafts is crucial for neurite outgrowth

Naoki Ichikawa1,2, Kazuhisa Iwabuchi3, Hidetake Kurihara4, Kumiko Ishii5, Yoshishide Kobayashi5, Takako Sasaki6, Nobutaka Hattori2, Yoshikuni Mizuno1,2, Kentaro Hozumi7, Yoshihiko Yamada7 and Eri Arikawa-Hirasawa1,2,*
1Research Institute for Diseases of Old Age, 2Department of Neurology, 3Institute for Environment and Gender Specific Medicine, and 4Department of Anatomy, Juntendo University School of Medicine, Tokyo, Japan
5Sphingolipid Functions Laboratory, RIKEN Frontier Research System, Saitama, Japan
6Shriners Hospitals for Children, Research Center, Portland, OR 97201, USA
7Laboratory of Cell and Developmental Biology, NIDCR, NIH, Bethesda, MD 20892, USA
*Author for correspondence (e-mail: ehirasawa@med.juntendo.ac.jp)

Summary
Laminin-1, an extracellular matrix molecule, promotes neurite outgrowth through the interaction of integrin and actin. Monosialoganglioside GM1 in the lipid rafts associates with and activates the NGF receptor TrkA, and enhances neurite outgrowth. However, the role of GM1 in laminin-1-induced neurite outgrowth was still unclear. Here, we describe that laminin-1 binds to GM1 through a carbohydrate moiety and a specific conformation of GM1, induces focal formation of large clusters of GM1, and enhances the relocation of TrkA in the membrane of dorsal root ganglion (DRG) and PC12 cells. We found that laminin-1-mediated clustering of GM1 causes the translocation and enrichment of β1 integrin in lipid rafts – where TrkA colocalizes with β1 integrin – and the activation of Lyn, Akt and MAPK to promote the outgrowth of neurites. Our results suggest that the binding of laminin-1 to GM1 facilitates the formation of a focal microdomain in the membrane, and enhances signal transduction that promotes neurite outgrowth by linking NGF-TrkA signaling with the laminin-integrin signaling pathways.

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Key words: GM1, Integrin, Laminin, Lipid rafts, Neurite outgrowth

Introduction
Neurite outgrowth is a key event in the differentiation of neuronal cells and is regulated by extracellular environment factors, including neurotrophic factors, such as nerve growth factor (NGF), and cell adhesion molecules, such as laminin. Laminin-1 is a heterotrimeric extracellular matrix protein composed of α1-, β1- and γ1-chains that is crucial for early basement-membrane assembly, embryonic implantation and development (Aumailley et al., 2005; Ekbloom et al., 2003; Li et al., 2002; Miner et al., 2004). In the nervous system, laminin-1 is expressed in the developing brain in a time- and region-specific manner (Miner and Yurchenco, 2004; Sanes, 1989). Laminin-1 is also expressed in the peripheral nervous system during development and in response to injury. It promotes neurite outgrowth in various neuronal cells (Kleinman et al., 1979; Kleinman et al., 1990; Reichardt and Tomasielli, 1991), and several active sites in laminin α1- and γ1-chains have been identified to be important for neurite outgrowth (Hager et al., 1998; Liesi et al., 2001; Nomizu et al., 1998; Powell et al., 2000; Richard et al., 1996). Binding of laminin-1 to integrins activates integrins through a conformational change that then promotes neurite outgrowth (Ivins et al., 2000). However, laminin-1-mediated neurite outgrowth requires NGF (Tucker et al., 2005). Neuronal cells undergo neurotigenesis upon the activation of NGF signaling via the Trk family of tyrosine protein kinase receptors, such as TrkA (Huang and Reichardt, 2001). NGF signaling causes neurite outgrowth in neuronal cells but the effect is not sufficient for rapid and robust neurite outgrowth, and laminin-independent activation of integrins enhances its potential. These independent pathways cooperatively activate Src in dorsal root ganglion (DRG) neurons (Tucker et al., 2005).

Sialic-acid-containing glycosphingolipids are abundant in neurons and have been implicated in neurogenesis. Among these glycosphingolipids, monosialoganglioside GM1 has an important role in neurogenesis, such as neurite formation, axon guidance and synapse formation, as well as in the formation of neuromuscular junctions (Ledeen et al., 1998; Nakai and Kamiguchi, 2002; Wu et al., 2007). GM1 is highly expressed on the plasma membrane of the neural cells and tightly associated with the NGF receptor TrkA (Mutoh et al., 1995; Nishio et al., 2004). Glycosphingolipids such as GM1 consist of hydrophobic ceramide and hydrophilic sugar moieties, and are implicated in cellular processes such as cell adhesion (Degroote et al., 2004); (Hakomori et al., 1998; Simons and Toomre, 2000). Essential functions of glycosphingolipids rely on their clustering with signal transducers in lipid rafts (membrane microdomains) (Hakomori et al., 1998; Simons and Toomre, 2000). Glycosphingolipid-enriched lipid rafts usually contain signal transducers such as Src family kinases (Iwabuchi et al., 1998a; Iwabuchi and Nagaoka, 2002; Iwabuchi et al., 1998b; Kasahara et al., 1997; Prinetti et al., 1999). Clustering of cell-surface receptors, signaling molecules and glycosphingolipids form focal lipid rafts in the cell membrane that transduce signals for various cellular processes (Kusumi et al., 2004; Yoshizaki et al., 2008).

In the immune system, the ligand-induced clustering of lipid rafts forms immunological synapses (Viola et al., 1999), and is required for transducing cellular signals for lymphocyte differentiation (Van...
Laethem and Leo, 2002). In neurons, the neuromuscular junction is formed by a unique set of molecules (Hoch, 2003). In both systems, the extracellular molecule agrin induces the local aggregation of specific molecules in the cell membrane (Bezakova and Ruegg, 2003). Although its molecular basis is not clear, synaptogenesis in the central nervous system (CNS) is also regulated by agrin (Bose et al., 2000). However, it is known that agrin inhibits but does not promote neurite outgrowth. During axonal outgrowth, the protrusion of neurites may also be regulated by lipid rafts in the plasma membrane, where specific molecules are enriched (da Silva and Dotti, 2002).

Laminin has been shown to bind several gangliosides from the brain (Laitinen et al., 1987). It has also been reported that sialic-acid residues on astrocytes regulate neuritogenesis by controlling the assembly of laminin matrices (Freire et al., 2004), where the removal of sialic-acid groups on the embryonic monolayer by neuraminidase treatment lead to the immediate release of matrix-associated laminin. In this study, we demonstrate that laminin-1 induces focal clustering of GM1 in the plasma membrane by directly binding to GM1, thereby causing TrkA and β1 integrins to accumulate, and activating signaling pathways to promote neurite outgrowth. Our results suggest that laminin-1 promotes neurite outgrowth by inducing the enrichment of β1 integrins and TrkA in GM1-enriched lipid rafts, in which both laminin-1-integrin and NGF-TrkA signaling pathways are linked and cooperatively enhanced.

Results
Laminin-1 induces clustering and colocalization of GM1 and TrkA
Monosialoganglioside GM1 is present in the lipid raft of the plasma membrane of neuronal cells and acts as a neuroprotective molecule that promotes dimerization and activation of NGF receptors upon treatment with NGF (Mutoh et al., 2000; Mutoh et al., 1995). Since laminin-1 promotes NGF-mediated neurite outgrowth, laminin-1 might be involved in the focal enrichment of GM1 in the plasma membrane. To investigate this possibility, we treated mouse embryo dorsal root ganglia (DRG) cells with NGF or NGF and laminin-1, and visualized endogenous GM1 using anti-GM1 antibody or cholera toxin B subunit (CTxB), which binds to GM1 and is often used to label GM1. Using confocal laser microscopy, we observed that laminin-1 induced strong focal clustering of endogenous GM1 (Fig. 1A). To analyze laminin-1-induced clustering of GM1 in detail, we labeled living DRG cells with fluorescently tagged GM1 (BODIPY-GM1) because CTxB is a pentameric molecule that can bind to five GM1 molecules (Middlebrook and Dorland, 1984) and might change the distribution of GM1 in the membrane (Middlebrook and Dorland, 1984). Under these conditions, BODIPY-GM1 is incorporated into the plasma

![Image](https://example.com/image.png)
Laminin-1 binds directly to GM1

Since laminin-1 induced the formation of large GM1 clusters in the rafts, we next investigated the interaction of laminin-1 with GM1 using a solid-phase binding assay in vitro and a bead assay in live cells. The solid-phase binding assay revealed that laminin-1 bound to GM1, whereas it did not bind to asialo-GM1 (GM1 lacking sialic acid; Neu5Acα2; Fig. 2A, Fig. 3), suggesting that the sialic acid of GM1 is important for GM1 binding to laminin-1. Binding of laminin-1 to GM2 and GM3 was substantially weaker than to GM1 (Fig. 2A). Since both GM2 and GM3 contain sialic acid, our results indicate that sialic acid alone is not sufficient for the specific binding of laminin-1. Furthermore, the binding of laminin-1 to GD1a and GD1b, which have the same sugar chain as GM1 but contain two sialic acids (Fig. 3), was substantially less than the binding to GM1 (Fig. 2A), which suggests that the conformation of GM1 is important for its binding to laminin-1. We also examined whether laminin-1 induced clustering of GM1 on the cell membrane by using a bead assay. Laminin-1-coated beads or transferrin-coated control beads were incubated with living DRG neuronal cells that had been labeled with BODIPY-GM1. We observed an aggregation of GM1 on the membrane mimicking endogenous GM1 (Pitto et al., 2000). Small clusters of BODIPY-GM1 on the membrane of NGF-primed living DRG cells started to form immediately after the addition of laminin-1 (data not shown) and large clusters became obvious 5 minutes after the addition of laminin-1 (Fig. 1B). To distinguish these clusters from signals caused by membrane ruffling, we labeled the membrane with the lipophilic probe 1,1′-dioctadecyl-3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI-C18), a widely used low-toxicity dye that labels cell membranes by inserting its two long hydrocarbon chains into the lipid bilayers. DiI-C18 did not cluster after laminin-1 treatment (Fig. 1B). The transferrin receptor (TIR) was used as a control of a nonraft protein (Chamberlain et al., 2001) and did not colocalize with GM1 in the presence of laminin-1 – analysed using confocal imaging and detergent-resistant membrane (raft) fractions (supplementary material Fig. S1A,B). These results indicate that laminin-1 specifically induces the focal clustering of GM1.

Since laminin-1-mediated promotion of neurite outgrowth requires NGF in many neuronal cells, we analyzed the localization of GM1 and NGF receptor TrkA in living DRG cells in the presence of laminin-1 (supplementary material Fig. S2). Florescence imaging and semi-quantitative analysis suggested colocalizations of GM1 and TrkA (supplementary material Fig. S2). To confirm clustering of GM1 molecules observed by confocal laser microscopy, we also analyzed the localization of GM1 on the membrane of DRG cells treated with NGF or NGF plus laminin-1 using electron microscopy. Gold particles to detect GM1 were localized close to each other and formed clusters when DRG cells were treated with NGF plus laminin-1 compared with NGF alone (Fig. 1C). We found clustering of GM1-gold particles even on the membrane where no ruffling was observed. These results indicate that laminin-1 induces clustering of GM1 on plasma membranes of neuronal cells.

**Laminin-1 binds to GM1**

Li et al., 2005). Since α-dystroglycan has sialic acid bound to the Galβ1 residue (Neu5Acα2-3Galβ1) (see Fig. 3) – a feature common to GM1, GM2 and GM3 – the LG4 subdomain might be a binding domain for GM1. We prepared recombinant LG4 to test its binding to GM1 and to other glycosphingolipids. We found that LG4 bound to GM1 but not to asialo-GM1 or lactosylceramide (LacCer) with a specificity similar to that of laminin-1 (Fig. 2B). However, LG4-binding activity was less than that of laminin-1 when compared with the molar ratio for their binding to GM1. In competition assays, LG4 inhibited binding of laminin-1 to GM1 (Fig. 2C), suggesting that LG4 contains sites for laminin-1 binding to GM1. To further delineate the binding site within LG4, we tested the activity of the 12-mer peptide AG73 within LG4. We found that AG73 bound to GM1-coated plates (5 μg/well). Each value is given as the mean (+ s.d.) of three experiments.

Since α-dystroglycan is also a receptor for laminin-1, this interaction is of particular interest. The LG4 subdomain of the laminin-α1 C-terminal globular domain contains several binding sites for heparin, heparan sulfate proteoglycans, dystroglycans and sulphatide (Andac et al., 1999; Harrison et al., 2007; Hoffman et al., 1998; Hozumi et al., 2006;
localization of β1 integrins. To examine whether β1 integrin is localized in lipid rafts, we analyzed lipid raft fractions prepared from PC12 cells (Fig. 4A). Treatment of PC12 cells with NGF alone increased TrkA in the raft fractions, a finding that is consistent with a previous report (Limpert et al., 2007). Treatment of PC12 cells with laminin-1 alone caused an increase of β1 integrin but not TrkA in the raft fractions (Fig. 4A). By contrast, treatment of PC12 cells with both laminin-1 and NGF markedly increased the amount of β1 integrin and TrkA in the lipid raft fractions (Fig. 4A), whereas the amount of the classic lipid-raft-associated protein flotillin-1 was not changed. Quantitative analysis of β1 integrin levels revealed a four-fold or 7.5-fold increase of β1 integrin in the raft fractions of PC12 cells that had been treated with laminin-1 alone or with laminin-1 plus NGF, respectively (supplementary material Fig. S4). These results indicate that laminin-1 induced the accumulation of β1 integrin in the lipid rafts and, together with NGF, enhanced its accumulation. To observe these phenomena in living cells, we next examined clustering of β1 integrin in DRG cells labeled with BODIPY-GM1 in the presence of laminin-1 (Fig. 4B). β1 integrin (red) formed clusters and colocalized with BODIPY-GM1 (green) in DRG cells after the addition of laminin-1 (Fig. 4B). These results suggest that laminin-1-induced clustering of GM1 accompanies the clustering of β1 integrins in the same lipid rafts of the membrane, consistent with our biochemical analyses.

To investigate the change in NGF-dependent signaling, we analyzed the activation of TrkA, Akt and MAPK in PC12 cells treated with NGF and/or laminin-1. NGF alone induced phosphorylation of TrkA, Akt and MAPK, but laminin-1 alone did not (supplementary material Fig. S5A). Treatment of cells with both NGF and laminin-1 enhanced phosphorylation of Akt and MAPK, whereas phosphorylation levels of TrkA were almost the same as those seen after the treatment with NGF alone (supplementary material Fig. S5A). Treatment with NGF and CTXb together did not increase phosphorylation of TrkA, Akt, and MAPK (supplementary material Fig. S5B). These results indicate that laminin-1 enhances the activation of Akt and MAPK in NGF-treated PC12 cells.

Laminin-1-induced clustering of TrkA in DRG cells observed by electron microscopy
To confirm the results obtained from the confocal laser microscopy, we analyzed the localization of TrkA on the membrane of DRG cells treated with NGF or NGF plus laminin-1 by electron microscopy. Gold particles to detect TrkA were localized close to each other and formed clusters when DRG cells were treated with NGF and laminin-1 compared with treatment with NGF alone (Fig. 5A). We evaluated clusters by counting the gold particles within a diameter of 100 nm. The number of clusters containing four or more gold particles were substantially increased when both NGF and laminin-1 were added, indicating that laminin-1 enhanced the clustering of TrkA (Fig. 5A). These results were consistent with those of our biochemical analysis (Fig. 4A) and the confocal laser microscopy (supplementary material Fig. S2). We also tested the localization of β1 integrin in the presence of laminin-1. β1 integrin (Fig. 5B, arrowheads, large particle) was visible as individual gold particles in the treatment with NGF alone (control). However, in the presence of laminin-1, β1 integrin (large gold particles) often colocalized with TrkA (small gold particles) (Fig. 5B). These results are consistent with our biochemical analyses, suggesting colocalization of β1 integrin and TrkA (Fig. 4).

Clustering of GM1 is essential for laminin-1-induced neurite outgrowth
We next examined whether the clustering of GM1 is essential for laminin-1-mediated neurite outgrowth, by using GM1 derivatives as inhibitors. We found that lyso-GM1, a derivative of GM1 that lacks fatty-acid moieties, inhibited laminin-1-mediated GM1 clustering and neurite outgrowth in DRG cells (Fig. 6A,B). There was no inhibitory effect on the clustering of GM1 or neurite outgrowth by lyso-lactosylceramide (lyso-LacCer) (Fig. 6A,B). Lyso-GM1, but not lyso-LacCer, inhibited the clustering of TrkA (Fig. 6A). Our observation that lyso-GM1 dispersed laminin-1-induced GM1 clusters is similar to the finding that lyso-GM3 disrupted GM3 clustering and inhibited GM3-mediated signal transduction in B16 melanoma cells (Iwabuchi et al., 2000). Lyso-GM2 and lyso-GM3 did not inhibit laminin-1-mediated neurite outgrowth in DRG and PC12 cells (data not shown), indicating that GM2 and GM3 are not involved in neurite outgrowth, in agreement with a previous study (Mutoh et al., 1998). These results suggest that the carbohydrate moiety of GM1 is important for the binding of GM1 to laminin-1 and that the clustering of GM1 is essential for neurite outgrowth. Moreover, lyso-GM1 also disrupted the
Laminin-1 binds to GM1 in lipid rafts

To further assess the significance of GM1 and β1 integrin in neurite outgrowth, we knocked down their expression in PC12 cells using specific small interfering RNA (siRNA). The effectiveness of siRNAs was first evaluated at different time points. GM1 expression was reduced within 24-48 hours after the transfection of siRNA targeting GM1 synthase (β1,3-galactosyltransferase). Laminin-1-induced neurite outgrowth was significantly reduced in GM1-knockdown cells (Fig. 7A), indicating that GM1 is essential for laminin-1-induced neurite outgrowth. β1 integrin expression was reduced in PC12 cells within 24-36 hours after the transfection of siRNA targeting β1 integrin (Fig. 7B). Under these conditions, laminin-1-mediated neurite outgrowth was reduced. Incubation with antibody against β1 integrin inhibited neurite outgrowth of PC12 cells (Fig. 7C). These results indicate that both GM1 and β1 integrin are required for laminin-1-induced neurite outgrowth.

Inhibition of Lyn reduced laminin-mediated neurite outgrowth in PC12 cells

Since Src family tyrosine kinases, such as Lyn, Fyn and Src, are found in lipid rafts (Galbiati, 2001) and are involved in NGF signaling, we investigated whether laminin-1-induced GM1 clustering activates these kinases. Laminin-1-induced neurite outgrowth was inhibited by the Src-family tyrosine kinase inhibitor PP1 but not by PP3, a structurally related molecule that does not inhibit Src family members (data not shown) compatible with a previous study (Tucker et al., 2005). Lyn was abundant in lipid raft fractions but was inactive...
because it was not detectable using the phospho-Y396 Lyn antibody without NGF (Fig. 8A). Treatment with NGF or laminin-1 induced activation of Lyn to some extent, but treatment with both NGF and laminin-1 substantially enhanced activation of Lyn (Fig. 8A). This activation occurred within 5 minutes after the treatment (Fig. 8A). CTxB, which forms a complex with GM1 and generates small aggregates of GM1, also induced phosphorylation of Lyn (Fig. 8B). The phosphorylation of Lyn induced by CTxB was less extensive than that of laminin-1, indicating that the aggregation of GM1 itself can activate the phosphorylation of Lyn (Fig. 8B). Further, inhibition of GM1 clustering using lyso-GM1 reduced the phosphorylation of Lyn in PC12 cells treated with NGF and laminin-1 (Fig. 8B). These results suggest that the clustering of GM1 is required for the activation of Lyn.

We observed that inhibition of clustering of GM1 by lyso-GM1 inhibited the protrusion of neurites from PC12 cells (supplementary material Fig. S6). However, knockdown of β1 integrin did not prevent neurites from forming but the processes were not elongated. β1 integrin might have a more important role in elongation than in formation of neurites. Next, we investigated the effect the knockdown of GM1 or β1 integrin had on laminin-1-induced neurite outgrowth. Knockdown of GM1 expression in PC12 cells using siRNA decreased phosphorylation of Lyn, but had no effect on Lyn protein (Fig. 8C). siRNA targeting β1 integrin also reduced phosphorylation of Lyn. These results suggest that both GM1 and β1 integrin are crucial for laminin-1-induced neurite outgrowth activity through activation of Lyn (Figs 7 and 8). To further address the significance of Lyn in neurite outgrowth, we knocked down the expression of endogenous Lyn in PC12 cells by using siRNA. RNA interference (RNAi) of Lyn reduced Lyn protein levels by more than 70% within 48 hours after transfection (Fig. 9A). Following Lyn knockdown in PC12 cells, laminin-1-mediated neurite outgrowth was significantly reduced.
(Fig. 9B,C), confirming that Lyn is an important signal molecule for laminin-1-induced neurite outgrowth.

**Discussion**

In this study, we have identified a crucial role for the ganglioside GM1 in laminin-1-induced signaling that leads to neurite outgrowth in PC12 cells and DRG neurons. We found that binding of laminin-1 to GM1 induces the aggregation of GM1 and promotes subsequent clustering of TrkA and β1 integrin in GM1-enriched lipid rafts, thereby stimulating downstream signaling (including Akt and MAPK) that promotes neurite outgrowth (supplementary material Fig. S5). Our results suggest that laminin-1 binding to GM1 promotes focal GM1 aggregation in the lipid rafts on the plasma membrane that facilitate the compartmentalization of microdomains, thereby acting as a signaling center for neurite outgrowth by linking and coordinating with NGF and integrin signaling pathways.

We found that laminin-1 binds to GM1 through a carbohydrate moiety of GM1. Sialic acid is required for the binding because asialo-GM1 (which lacks sialic acid) did not bind to laminin-1 (Fig. 3). GD1a and GD1b, which contain the same sugar chains as GM1 but have two sialic acids, bound to laminin-1 less strongly than GM1, suggesting that the conformation of GM1 for laminin-1 binding is important. Laminin-1 bound to GM2 through the same carbohydrate moiety, but its binding was less extensive than to GM1. This suggests that the additional Galβ1 residue at the 5’ end of the carbohydrate moiety of GM1 is required for effective binding to laminin-1. Lyso-GM1, which lacks a fatty acid, inhibited laminin-1-mediated neurite outgrowth. These results suggest that the extent of the aggregation and the conformation of GM1 are important for the effective clustering of signaling molecules and the subsequent promotion of neurite outgrowth by laminin-1. Laitinen et al. have demonstrated – using the thin-layer chromatogram (TLC) overlay method – that binding of GD1a to laminin-1 is stronger than that of GM1 to laminin-1 (Laitinen et al., 1987). In our experiments, binding of GM1 to laminin-1 was stronger than that of GD1a to laminin-1 (Fig. 2). This difference is probably due to the difference in assay systems. In the TLC assay, they used polyisobutylmethacrylate to fix gangliosides and coordinating with NGF and integrin signaling pathways.

The LG4 module of laminin α1 contains major binding sites for heparin, sulfatide, syndecans and α-dystroglycan (Harrison et al., 2007; Hoffman et al., 1998; Hozumi et al., 2006; Li et al., 2005). We showed that LG4 is one of the binding sites of laminin-1 for GM1. Because the binding of LG4 to GM1 is less effective than that of laminin-1 – the latter has probably multiple binding sites for GM1 – or LG4 binds to GM1 in cooperation with other sites of laminin-1 for effective GM1 binding. We also found that the AG73 sequence within LG4 is an active binding site. The ability of LG4 to bind to different carbohydrates suggests that laminin-1 can exert different functions in tissues and in development by interacting with tissue- or site-specific carbohydrate moieties. Our live-cell images showed rapid focal clustering of TrkA and β1 integrin colocalized with GM1 in neuronal cells upon the addition of laminin-1. No clustering of β1 integrin was induced when clustering of GM1 was inhibited through lyso-GM1 (Fig. 6), suggesting that clustering of GM1 is a prerequisite for the enrichment of β1 integrin in the lipid rafts. However, enrichment and activation of integrin(s) are essential for laminin-1-mediated neurite outgrowth (Figs 6-8) (Ivins et al., 2000), suggesting that laminin-1 initially binds to GM1 and to induce its clustering, which then promotes the enrichment of TrkA in the lipid rafts. The clustering of GM1 is also likely to facilitate the relocation of β1 integrins on the plasma membrane, which promotes the interaction of laminin-1 and integrin in the lipid rafts. Subsequently, TrkA and integrin activate their signaling pathways in a cooperative manner (Fig. 10). Since laminin-1 self-assembly has been reported (Yurchenco et al., 1992), local laminin-1 aggregation might strengthen in focal GM1 aggregation structures in lipid rafts. Laminin-1-NGF-mediated neurite outgrowth requires the activation of the Src family of tyrosine kinases (SKF), which results in the activation of downstream signaling intermediates, such as FAK and Akt (Tucker et al., 2008). We also demonstrated in this study that laminin-1 and NGF activate Akt and MAPK (supplementary material Fig. S5). Our results that PC12 cells in which Lyn was knocked down failed to respond to neurite outgrowth by laminin-1 and NGF (Fig. 8) suggest that Lyn is a primary SKF molecule that requires laminin-1–NGF-promoted neurite outgrowth of neuronal cells, because other SFKs are present in the lipid rafts of cells.
In our biochemical analysis, we prepared detergent-resistant membrane (R in Fig. 8A), which contains lipid rafts (Brown and London, 1997). It has been reported previously that GM1 is mainly localized in DRM fractions (Russelakis-Carneiro et al., 2004). Indeed, we also found that GM1 was present predominantly in lipid-raft fractions, not in non-raft fractions (supplementary material Fig. S1). Glycosphingolipid-enriched lipid rafts that contain signal transducers, such as SFKs and small G proteins (Iwabuchi and Nagoaka, 2002; Prinetti et al., 1999), are probably involved in glycosphingolipid-dependent cellular functions (Kasahara et al., 1997; Prinetti et al., 2000). We found that the Lyn protein is present in DRM fractions of PC12 cells that had not been stimulated with laminin-1 (Fig. 8A). However, phosphorylation levels of Lyn were very low without laminin-1 treatment, even if NGF present (Fig. 8A). CTxB, which causes little GM1 aggregation, increased phosphorylation of Lyn to some extent, but laminin-1 was required for high levels of activation Lyn in NGF-primed PC12 cells (Fig. 8B). This activation is required for GM1 to aggregate because lysogalactosyl-sulfatide in order to initiate the assembly of the basement membrane for dystroglycan signaling in Schwann cells and fibroblasts (Li et al., 2005). The laminin-GM1 interaction and GM1 clustering in neuronal cells might provide a platform in the plasma membrane for the enrichment of integrin in the membrane similar to the cellular processes of these cells.

Different types of lipid rafts may – depending on the signal – form. For example, laminin-1 and agrin are basement-membrane components that bind α5-dystroglycan through their C-terminal globular domain. However, they have opposite effects in the neuronal system. Laminin-1 promotes neurite outgrowth, whereas agrin inhibits it (Campagna et al., 1995; Mantych and Ferreira, 2001). Agrin is a heparan-sulfate proteoglycan, and its inhibition of neurite outgrowth can be either glycosaminoglycan-dependent or -independent (Baerwald-de la Torre et al., 2004). Agrin induces a redistribution of the lipid rafts, aggregation of signaling molecules, and the creation of signaling domains in both immune and neuronal systems (Bezakova and Ruegg, 2003; Khan et al., 2001). However, unlike laminin-1, agrin does not bind GM1 (data not shown) or β1 integrins. The difference in activity between agrin and laminin-1 in the neuronal system might be – at least in part – be owing to the integrin α5β1, which is located in LG4 at the C-terminal side from the AG73 site has been identified as a binding site for integrin α5β1 integrin (Hozumi et al., 2006). Laminin-1 and laminin-2 bind the glycolipid galactosyl-sulfatide in order to initiate the assembly of the basement membrane for dystroglycan signaling in Schwann cells and fibroblasts (Li et al., 2005). The laminin-GM1 interaction and GM1 clustering in neuronal cells might provide a platform in the plasma membrane for the enrichment of integrin in the membrane similar to the cellular processes of these cells.

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Materials and Methods
Cell culture and neurite-outgrowth assay
Rat PC12 cells were cultured as described previously (Weeks et al., 1998). DRG cells were obtained from E14.5 mouse embryos and cultured in labeling buffer [DMEM/F12 with 1% N2 supplement (Gibco), 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 100 ng/ml of NGF (Roche Applied Science, Indianapolis, IN)] as described (Ivins et al., 2000). The neurite outgrowth assay was performed as described previously (Ishikawa et al., 2005). DRG cells were pretreated with 2.5 μM lyso-GM1 (Matreya, Inc.) or 2.5 μM lyso-lactosylceramide (LacCer) (Matreya, Inc.) for 30 minutes. The cells were incubated with 5 μg/ml of laminin-1 for 24 hours and then stained with anti-neurofilament antibody. The length of neurites in DRG cells

![Figure 9](image-url) Depletion of endogenous Lyn inhibits laminin-induced neurite outgrowth. (A) PC12 cells were transfected with vector expressing EGFP and siRNA targeting Lyn. Lyn expression was analyzed by western blotting 24 hours and 48 hours after transfection. (B,C) At 24 hours after siRNA transfection, neurite outgrowth was analyzed and the percentage of EGFP-expressing cells with neurite outgrowth was determined as described in Materials and Methods. Data indicate the mean ± s.d. of triplicate results (*P<0.05; two-sided t-test). Bars, 50 μm. (Fig. 9. Depletion of endogenous Lyn inhibits laminin-induced neurite outgrowth. (A) PC12 cells were transfected with vector expressing EGFP and siRNA targeting Lyn. Lyn expression was analyzed by western blotting 24 hours and 48 hours after transfection. (B,C) At 24 hours after siRNA transfection, neurite outgrowth was analyzed and the percentage of EGFP-expressing cells with neurite outgrowth was determined as described in Materials and Methods. Data indicate the mean ± s.d. of triplicate results (*P<0.05; two-sided t-test). Bars, 50 μm.)
was measured using confocal laser microscopy (Carl Zeiss LSM510 instrument) and imaged software. PC12 cells were plated with DMEM/F12 containing 100 ng/ml of NGF, incubated for 24 hours and fixed with 20% formalin. Then, the number of cells that had processes equal to or more than two times the cell-body diameter was determined. For anti-β1-integrin antibody inhibition, PC12 cells were preincubated with 10 μg/ml of anti-β1 integrin antibody (65E6) at room temperature for 20 minutes. Cells were then incubated for 24 hours and fixed. Trypan Blue staining confirmed that all inhibitors at the concentrations used in this study were nontoxic.

Recombinant LG4 and synthetic peptide AG73
Recombinant LG4 (Leu2683-Pro2874 of mouse laminin α1; GenBank accession number J04064) was prepared from human embryonic kidney (HEK) cells expressing EBNA1 (HEK293-EBNA1) cells transfected with pCEP4 PurAL.G4. The expression vector pCEP4 PurA originated from pCEP4 (Invitrogen) and is a modified version of pCEP4-Po (Kohfeldt et al., 1997). pCEP4 PurA contains the cytomegalovirus (CMV) promoter and enhancer, the B4M0 signal peptide, a 6His-tag, polycloning sites, and the tk-Puramycin resistance gene. cDNA encoding the mouse laminin α1 LG4 domain was cloned into the HindIII and NotI sites of pcDNA4 Pur (pcDNA4 Pur-AG73). Purified proteins were dialyzed against PBS and quantified using a BCA protein assay kit with bovine serum albumin as a standard (PIERCE). The purified LG4 protein appears as a single band on an SDS-PAGE gel under both reduced and nonreduced conditions.

Confocal detection of GM1 and cell-surface receptors
DRG cells in labeling buffer were plated on a poly-L-lysine-coated glass dish and preincubated with 2.5 μM lyso-GM1 or 2.5 μM lyso-LacCer for 30 minutes. 500 nM BODIPY-GM1 (Molecular Probes) was added to the cells for 5 minutes on ice. Dil-C18 (5 μM), Alexa-Fluor-546-conjugated rabbit anti-TrkA antibody, rabbit anti- transferrin-receptor antibody or rat anti-β1 integrin antibody was added to the cells and incubated for 10 minutes at 37°C. Cells were washed with warmed labeling buffer and incubated with 5 μg/ml of laminin-1. DRG cells were analyzed by confocal microscopy (model LCS; Leica) with a 100×/1.3 oil immersion objective (Leica) at 37°C in 5% CO2.

Antibodies
Antibodies against laminin-1 (L9393), neurofilament 200 (N4142) (Sigma-Aldrich); β1 integrin (E9GT7), fibronectin-1 (clone 1810) (BD Pharmingen); β1 integrin (M-106), actin (C-4), Lyn (44 and H-6), TrkA (763) (Santa Cruz); β1 integrin (686) (Chemicon); TrkA (06-574) (Upstate Biotechnology); phosphorulated Lyn (Y396) (EP503Y) (Eptomics, Inc.) were obtained commercially. Rabbit anti-laminin-α1 LG4-3 antibody and anti-laminin-α1 LG4-5 antibodies have been described previously (Ort et al., 1982; Sceele et al., 2005). Mouse anti-GM1 antibody was from Nobuhari Yuki (Dokkyo Medical University, Tochigi, Japan).

Binding of laminin-1 to glycosphingolipids
Bound glycolipids were measured by an enzyme-linked immunosorbent assay (ELISA). In brief, 5 μg of each glycosphingolipid (Matreya, Inc.) well were dried at room temperature. The coated wells were blocked with 1% BSA in PBS. Then, 1 ng/well laminin-1 and 1 ng/well LG4 in PBS (+) (PBS plus 1 mM CaCl2 and MgSO4) was added to the wells for 1 hour, followed by treatment with rabbit anti-laminin-1, rabbit anti-LG4-5 or rabbit anti-LG1-3 antibodies. The contents of the wells were then treated with HRP-conjugated anti-rabbit IgG or, in the case of biotin-A73, HRP-conjugated streptavidin (Amersham, Inc.). TMB (3,3′,5,5′-tetramethylbenzidine) solution was added to the wells, and then 2 M H2SO4 was added. Absorbance (OD450) was measured using a fluorescence plate reader with a bottom-reading system (ARVO, Perkin Elmer).

Immunoelectron microscopy
DRG cells were stimulated with 5 μg/ml of laminin-1 for 10 minutes and fixed with 4% paraformaldehyde (PFA) in PBS. Cells were washed and treated with blocking buffer (5% goat serum, 1% BSA, and 0.05% saponin in PBS) for 1 hour, and incubated with mouse anti-GM1 or rabbit anti-TrkA antibody and/or rat anti-β1 integrin antibody in blocking buffer overnight at 4°C. Next, cells were incubated with goat anti-mouse IgG coupled to 5-nm gold particles or goat anti-rabbit IgG coupled to 3-nm gold particles and/or goat anti-rat IgG coupled to 10-nm gold particles (British BioCell) in PBS containing 0.05% saponin (Calbiochem) for 1 hour. After immunostaining, the cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The cells were postfixed with 1% OsO4 in PBS for 1 hour at 4°C, dehydrated through a graded series of ethanol, and embedded in epoxy resin. Ultrathin sections were stained with 4% uranyl acetate and lead citrate and then examined with a JEM1230 (JOEL) electron microscope.

Immunoprecipitation and immunoblot analysis
PC12 cells were stimulated with either 20 μg/ml of cholera toxin B subunit (CTxB; List Biological Lab., Inc.) or 5 μg/ml of laminin-1 and washed with cold PBS. Cells were then homogenized in lysis buffer (20 mM HEPES, 10% glycerol, 1% NP-40, protease inhibitor mixture (Roche), 1 mM Na3VO4, and 2 mM NaF, pH 7.4) at 4°C for 30 minutes. After centrifugation, the supernate was incubated with anti-Lyn antibody at 4°C overnight and added to proteinG-Sepharose beads (GE Healthcare) at 4°C for 2 hours. The beads were washed with lysis buffer, added to NuPAGE LDS sample buffer (Invitrogen) containing 50 mM dithiothreitol (DTT) and boiled. Immunoblotting was performed using antibody against phosphorylated Lyn.

Isolation of lipid rafts
PC12 cells that had been pretreated with 100 ng/ml of NGF were incubated with 5 μg/ml of laminin-1 for 10 minutes. Cells were then resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease inhibitor mixture, 1 mM Na3VO4, and 2 mM NaF, pH 7.4) and incubated at 4°C for 20 minutes. After centrifugation, the supernate was incubated with anti-Lyn antibody at 4°C overnight and added to proteinG-Sepharose beads (GE Healthcare) at 4°C for 2 hours. The beads were washed with lysis buffer, added to NuPAGE LDS sample buffer (Invitrogen) containing 50 mM dithiothreitol (DTT) and boiled. Immunoblotting was performed using antibody against phosphorylated Lyn.

Transfection and siRNA
PC12 cells were transfected with siRNA and pAcGFPI-C1 vector or pHcRed1-C1 vector (Clontech) using a Nucleofector system (Amaza Biosystems) according to the manufacturer’s instructions. siRNAs targeting GM1, β1 integrin or Lyn were obtained from HP GenomeWide siRNA (Qagen). We routinely observed a transfection efficiency of >50% in PC12 cells.

Statistical analysis
All the experiments were performed at least three times and yielded similar results. Statistical data are given as the mean ± s.d. Differences were analyzed using the two-sided Student’s t-test with unequal variances.
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Laminin-1 binds to GM1 in lipid rafts

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