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ORIGINAL ARTICLE

A human IgM signals axon outgrowth: coupling lipid raft to microtubules

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Abstract

Mouse and human IgMs support neurite extension from primary cerebellar granule neurons. In this study using primary hippocampal and cortical neurons, we demonstrate that a recombinant human IgM, rHIgM12, promotes axon outgrowth by coupling membrane domains (lipid rafts) to microtubules. rHIgM12 binds to the surface of neuron and induces clustering of cholesterol and ganglioside GM1. After cell binding and membrane fractionation, rHIgM12 gets segregated into two pools, one associated with lipid raft fractions and the other with the detergent-insoluble cytoskeleton-containing pellet. Membrane-bound rHIgM12 co-localized with microtubules and co-immuno precipitated with β3-tubulin. rHIgM12-membrane interaction also enhanced the tyrosination of α-tubulin indicating a stabilization of new neurites. When presented as a substrate, rHIgM12 induced axon outgrowth from primary neurons. We now demonstrate that a recombinant human mAb can induce signals in neurons that regulate membrane lipids and microtubule dynamics required for axon extension. We propose that the pentameric structure of the IgM is critical to cross-link membrane lipids and proteins resulting in signaling cascades.

Keywords: axon, IgM, lipid raft, membrane, microtubule.

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Neurons elaborate axons and dendrites by regulating assembly of their cytoskeletons (Hoogenraad and Bradke 2009). Developing neurons extend multiple neurites. The fastest-elongating neurites develop into axons, whereas the others extend more slowly and differentiate into dendrites (Dotti *et al.* 1988; Goslin and Banker 1989). During this process of polarized axon outgrowth, signaling cascades guide axons to their targets (Barnes and Polleux 2009). Environmental factors activate signaling pathways that converge on cytoskeleton character and dynamics (Lowery and Van Vactor 2009) regulating axon outgrowth.

Many studies of neuron differentiation have focused on the actin cytoskeleton, but microtubules are emerging as another key player in axon outgrowth (Witte and Bradke 2008). Microtubules constitute the main architecture of the neuronal cell body, the shaft of processes and the growth cone central domain (Forscher and Smith 1988; Conde and Caceres 2009). Microtubules extend from the centrosome (Higginbotham and Gleeson 2007) to form bundled microtubule fasciculations in the cell processes that defasciculate within the growth cones. The role of microtubules in neurons has recently expanded from merely structural to an active role in the process of neuron differentiation. Exploring how signal cascades regulate microtubules may lead to important insights into axon outgrowth and regeneration.

Lipid raft microdomains serve as scaffolds for membrane-signaling molecules distributed along the neuron (Lingwood and Simons 2010). Membrane microdomains mediate and control the cell's response to temporally and spatially changing signals (Golub *et al.* 2004). Many neural cell adhesion and trans-membrane molecules involved in signal transduction contain immunoglobulin-like (Ig) motifs (Volkmer *et al.* 1992; Chothia and Jones 1997; Shapiro *et al.* 2007) and are coupled to signaling mediated by membrane domains (Niethammer *et al.* 2002). Antibodies can recruit T- and B-cell receptors to lipid raft microdomains, and this action is regulated by F-actin (Gupta *et al.* 2006;

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Abbreviations used: CTB, cholera toxin B; DIV, days *in vitro*; F-actin, Filamentous actin; PDL, poly-D-lysine; PS-NCAM, poly-sialylated neural cell adhesion molecule.

Chentouf *et al.* 2007), indicating that membrane domains can be linked to the underlying cytoskeletons. However, little is known about how membrane domains mediate signaling events to regulate microtubule motility. The microtubule-dependent signaling pathways leading to axon outgrowth and stabilization are far from clear.

In the current study, we show that a recombinant human monoclonal IgM, rHIgM12, when presented as a substrate, enhanced axon outgrowth in hippocampal neurons. Membrane-bound rHIgM12 clustered cholesterol and ganglioside GM1 within the membrane of these neurons. Following sucrose-gradient fractionation of neuronal membranes decorated with rHIgM12, the IgM was present, in part, in lipid-raft fractions containing caveolin-1. Membrane-bound rHIgM12 could pull down β3-tubulin and increased the level of α-tubulin tyrosination. These data support the hypothesis that pentameric IgMs bind to lipid rafts and cross-link signaling molecules on the neuronal membrane generating asymmetric anchoring of microtubules that promotes axon extension. These mAbs also provide useful reagents to study the role of membrane domain control of cytoskeleton assembly.

Materials and methods

Recombinant human IgM 12 (rHIgM12)

rHIgM12 was expressed in CHO cells (GibcoBRL, cat# 11619). Plasmids expressing the heavy and light chain coding sequences for the predominant antibody expressed in the serum of Waldenstrom's macroglobulinemia patient 12 were co-transfected along with a human J-chain transgene into CHO-S cells. The resulting CHO cells were selected with increasing doses of methotrexate, and a stable clone that produced the antibody as measured by ELISA was sub-cloned and expanded. rHIgM12 in culture supernatant was purified by chromatography to 97% as measured by HPLC analysis.

Cell culture and neurite outgrowth assay

Primary hippocampal neurons were prepared from FVB mice (The Jackson Laboratory, ME, USA). On embryonic day 15, hippocampal neurons were dissociated in trypsin-EDTA and plated on poly-D-lysine (PDL), laminin (50 μg/mL) plus PDL, rHIgM12 or control human IgM (50 μg/mL) substrates coated nitrocellulose membrane glass coverslips. Neurons were grown in neurobasal medium containing 2% (v/v) B27 supplement and assayed for neurite outgrowth 12 h after seeding. Neurons were fixed with 4% paraformaldehyde and stained with anti-β3-tubulin antibody. Filamentous actin (F-actin) was labeled with Texas-Red phalloidin and nuclei was labeled with DAPI. Neurite length in digital images was measured using Image J software (National Institute of Health, NIH), processed with Excel (Microsoft) and analyzed statistically with Prism (GraphPad Software Inc., San Diego, CA, USA) (Xu *et al.* 2009). A stage-3 neuron was defined as a neuron with multiple neurites where the longest neurite (Dotti *et al.* 1988), determined as an axon by *Tau*1 positive staining, was at least 2 times the length of the second longest neurite. *Tau*1 labeling was enriched in the distal segments of the axon. Stage-2 neurons were defined as those with multiple symmetric neurites. Animal care and the experimental protocols were approved by Mayo Clinic Institutional Animal Care and Use Committee (IACUC) and Research Committee.

Immunostaining, immunoprecipitation and sucrose density gradient fractionation of primary cultured neurons

E15 hippocampal neurons were seeded on PDL plus laminin (4 μg/mL)-coated glass coverslips. Neurons of 1–3 days *in vitro* (DIV) were immuno-stained either live on ice for surface molecules and/or fixed with 4% paraformaldehyde and then permeabilized in buffer containing 0.2% Triton X-100 to label intracellular components. Images were collected using an Olympus upright microscope equipped with a 37 Mb digital SPOT camera and processed with Photoshop (Adobe). The distribution of neuron-bound rHIgM12 in membrane fractions was determined using ultracentrifugation in a non-continuous sucrose gradient. Briefly, rHIgM12 (10 μg/mL) was allowed to bind to live DIV7 cortical neurons at 4°C for 30 min. Cells were then lysed in ice-cold lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and protease inhibitor cocktail) for 30 min. The neuronal lysates were mixed with an equal volume of 100% (w/v) sucrose, the mixture was transferred to a centrifuge tube, and 8 mL of 35% sucrose and 3.5 mL of 5% sucrose overlaid sequentially. After centrifugation at 2×10^5 *g* for 20 h at 4°C, six fractions (2 mL of each) were collected from the top of the gradient. Each fraction and pellet were dissolved in sodium dodecyl sulfate-sample buffer and subjected to western blotting. For co-immunoprecipitation of rHIgM12 with cytoskeleton protein, rHIgM12 was allowed to bind to DIV7 live cortical neurons at 4°C for 30 min and then lysed in buffer containing 0.5% NP-40 (Sigma, St Louis, MO, USA). rHIgM12 was captured using protein L-agarose beads and anti-β3-tubulin with protein G-resin (Thermo). Live neuron extraction and fixation were performed in buffer containing 60 mM Pipes, 25 mM Hepes, 5 mM EGTA, 1 mM $MgCl_2$, 4% paraformaldehyde and 0.1% Triton X-100 (Witte *et al.* 2008).

Antibodies and other reagents

Anti-β3-tubulin (Promega, Madison, WI, USA); anti-actin, anti-acetylated tubulin, EDTA, poly-D-lysine, methyl-β-cyclodextrin and filipin (Sigma, St Louis, MO, USA); anti-*Tau*1, anti-tyrosinated tubulin, anti-caveolin-1 and anti-transferrin receptor (Millipore Corporation, Bedford, MA, USA); Texas Red-phalloidin, cholera toxin B (CTB, Alexa Fluor 594), Neurobasal Medium and B27 (Invitrogen, Carlsbad, CA, USA); anti-β-actin, anti-α-tubulin (Cell Signaling Technology, Danvers, MA, USA); anti-poly-sialylated neural cell adhesion molecule, 5A5 (Hybridoma Bank, University of Iowa); protease inhibitor tablet (Roche Molecular Biochemicals, Indianapolis, IN, USA); EDO-P4: ceremide glycotransferase inhibitor (James Shayman, University of Michigan) and Mouse IgM, A2B5 (Eisenbarth *et al.* 1979).

Results

rHIgM12 regulates neuronal membrane lipid kinetics

Screening a human monoclonal gammopathy sera bank, we identified human IgM antibodies (sHIgM) that bound to the surface of neurons and supported neurite outgrowth from

primary cerebellar granule cells (Warrington *et al.* 2004). Enhanced neurite outgrowth was observed when the sHIgMs were presented as substrates. We hypothesized that neuron-binding IgMs, when presented as substrates, contact membranes at the point of cell adhesion and induced asymmetric signals across neuronal membranes akin to neural adhesion molecules. Bath-applied IgMs can interact with neuronal membranes randomly and did not promote neurite extension in granule cells but may impart other, less obvious signals. As the study with granule cells was performed, a recombinant form of the IgM derived from patient 12, rHIgM12, was synthesized. This recombinant human IgM also bound to the surface of neurons. Of significance, a single peripheral dose was found to alter activity in mice with long-term demyelination and neurologic dysfunction (Rodriguez *et al.* 2009).

This study explores the binding and signaling of this recombinant human IgM in a second type of primary neurons isolated from the hippocampus. When neurons were immuno-labeled with rHIgM12 after being fixed with 4% paraformaldehyde at 37°C without permeabilization, rHIgM12 evenly labeled the neuronal surface in a pattern similar to that of CTB and filipin, that bind to ganglioside, GM1 or cholesterol, respectively (Shogomori and Futerman 2001) (Fig. 1a). In contrast, when live neurons were first treated with bath-applied rHIgM12 at 37°C for 30 min, and then

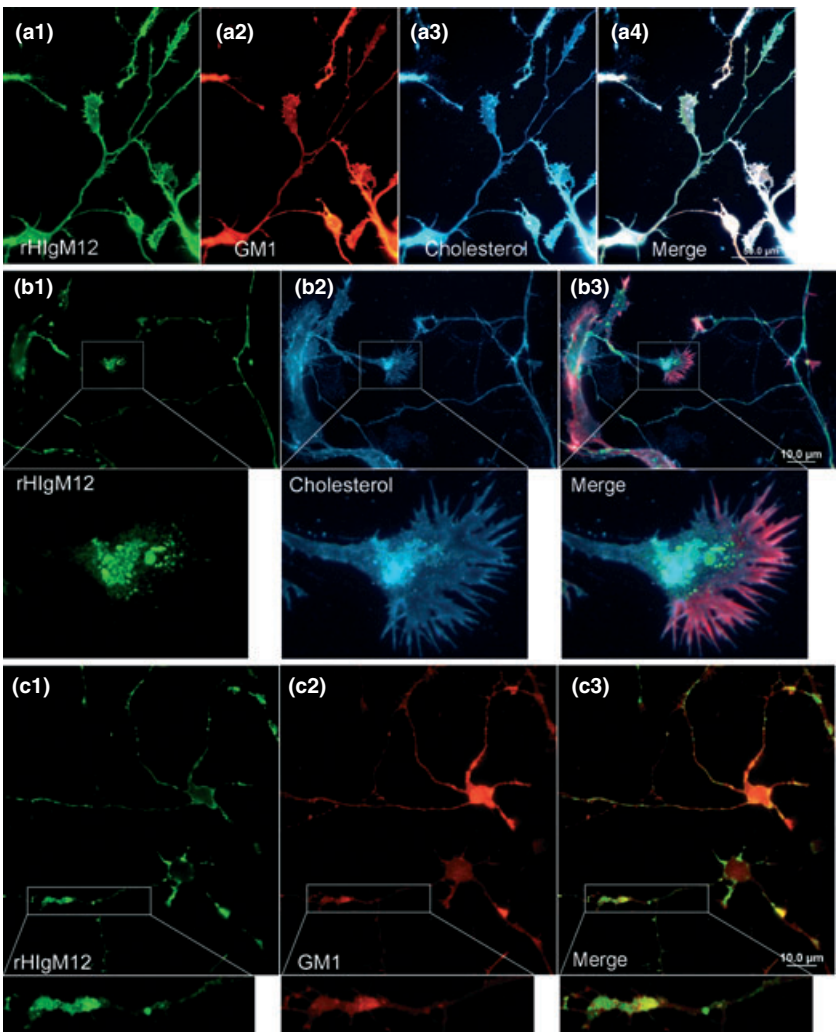

Fig. 1 rHIgM12 treatment induces the clustering of neuronal membrane lipids. (a) DIV3 hippocampal neurons were fixed and then triple stained with rHIgM12, CTB and filipin. rHIgM12 (a1, green) evenly labeled the neuronal surface, with a pattern similar to that of GM1 (a2, red) or cholesterol (a3, blue) indicated by CTB or filipin, respectively. (b–c). In contrast, when live neurons were treated with rHIgM12 for 30 min at 37°C, membrane-bound rHIgM12 aggregated on the neuronal surface (b1–c1, green). Treatment with rHIgM12 resulted in clustering of both cholesterol (b2, blue) and GM1 (c2, red), which co-localized with the aggregated rHIgM12 (b3–c3). Lower panels in the bottom of b & c are higher magnification of the boxed regions, showing the colocalization of aggregated rHIgM12 (green) with clustered cholesterol (blue) or GM1 (red) in the growth cone region. Scale bar in a = 50 μm; b & c = 10 μm.

fixed and double labeled with anti-human IgM secondary antibodies and filipin or CTB, a reorganization of membrane lipids had occurred (Fig. 1b and c). rHIgM12 appeared aggregated into 'patch'-like structures (Fig. 1b1 and c1) which was not observed in the pre-fixed neurons (Fig. 1a1) or live neurons treated with a control human IgM that does not bind to neurons (data not shown). Cholesterol (Fig. 1b2) and GM1 (Fig. 1c2) were also clustered at the membrane. Aggregated rHIgM12 co-localized almost perfectly with clustered cholesterol (Fig. 1b1 and b3) or GM1 (Fig. 1c1 and c3), especially within the growth cone central domain (Fig. 1b and c, high magnification), but not within the growth cone periphery. These results indicated that rHIgM12 applied to living neurons induces membrane lipid redistribution.

The binding of IgMs to cells may trigger Fc-receptor mediated internalization (Shibuya *et al.* 2000). To determine whether membrane-bound rHIgM12 was internalized upon binding, isolated hippocampal neurons were treated with rHIgM12 in the cold immediately after dissociation, and rHIgM12 distribution was analyzed 2–4 h after cell plating using an anti-human IgM secondary antibody (Fig. 2).

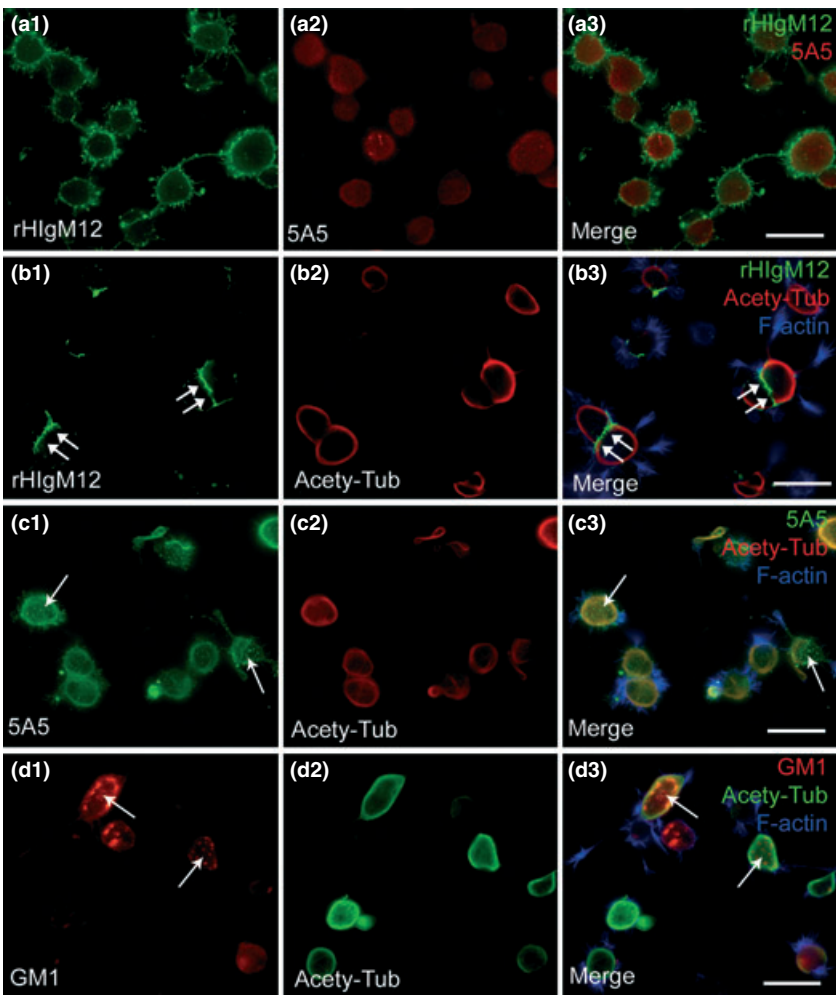

Fig. 2 rHIgM12 aggregates on neuronal surfaces but is not endocytosed. Acutely isolated hippocampal neurons were treated with rHIgM12, anti-PS-NCAM, 5A5 or CTB at 4°C before plating. The attached neurons were fixed 2–4 h after seeding and further stained with anti-human IgM, anti-acetylated α-tubulin (Acety-Tub) antibody and Alexa Fluor 350 phalloidin. (a) Hippocampal neurons differentiating *in vitro* first sent out lamellipodial and filopodial protrusions from the soma. On the focal planes where neurons attached to substrates, rHIgM12 labeled the filopodia and lamellipodia in the periphery (a1 and a3, green), whereas the mouse monoclonal antibody, 5A5, mainly stained the soma (a2 and a3, red). (b) The pre-labeled rHIgM12 (green) clustered along the outsides of microtubule rings (red) indicated by acetylated tubulin staining. In neurons that interacted with each other, rHIgM12 localized between the interfaces of the neurons (arrows). (c & d) In contrast, both 5A5 (c, green) and GM1 (d, red, stained by CTB) were redistributed to co-localize with and/or inside to the microtubule rings (arrows). These data indicated that membrane-bound rHIgM12, instead of being endocytosed, aggregated on neuronal surfaces, whereas both 5A5 and CTB, upon binding neuronal membranes, were internalized and transported along with microtubules. Scale bar = 20 μm.

Immediately after plating hippocampal neurons sent out lamellipodia and filopodia from the soma (Fig. 2a). At the focal plane where neurons contacted the substrate, rHIgM12 was found to be enriched in the filopodia and lamellipodia in the periphery (Fig. 2a1 and a3). In contrast, the mouse monoclonal antibody, 5A5, directed against poly-sialylated neural cell adhesion molecule (PS-NCAM), bound primarily at the soma (Fig. 2a2 and a3). By 2–4 h after plating of neurons, the membrane-associated rHIgM12 localized specifically at the interfaces of the neurons, determined by co-labeling for acetylated tubulin (Fig. 2b).

These observations indicated that, instead of being endocytosed, membrane-bound rHIgM12 shifted to cluster on the neuronal surface. In contrast, when cells were pre-labeled with IgM, 5A5 (Fig. 2c) or CTB (Fig. 2d) that bind PS-NCAM or GM1, these molecules were redistributed over time to co-localize with and/or inside microtubule rings, suggesting that 5A5 and CTB were internalized and transported along with microtubules.

rHIgM12 binds to lipid rafts

Our observations raised the possibility that rHIgM12 binds to lipid-raft microdomains. As neuronal membranes are enriched in cholesterol and glycosphingolipids with high melting temperatures (Tm) (Samsonov *et al.* 2001), decreasing the membrane temperature below that of the Tm may facilitate the visualization of lipid raft and associated molecules. To test this hypothesis, hippocampal neurons in culture were cooled to 4°C, then immuno-labeled with reagents. rHIgM12 bound to the membrane in large punctate structures, whereas CTB was evenly distributed (Fig. 3b). In contrast, when neurons were first fixed at 37°C then immuno-labeled with rHIgM12, the IgM distributed evenly on the neuronal membranes and partially co-localized with CTB (Fig. 3a). Low temperatures affected the distribution of rHIgM12-bound molecules more than that of GM1. We asked whether cholesterol depletion, which disrupts lipid raft integrity, would alter rHIgM12 binding to the membrane (Chichili and Rodgers 2009). Hippocampal neurons in culture were treated with 5 μM of methyl-β-cyclodextrin for 30 min at 37°C to deplete cholesterol (Ko *et al.* 2005) and then cooled to 4°C, fixed and then immuno-labeled with rHIgM12 and CTB. rHIgM12 bound as tiny punctate structures, which co-localized with CTB on the cholesterol-depleted membranes (Fig. 3c). These results indicated that rHIgM12-bound molecules and GM1 partitioned to the same membrane microdomains upon cholesterol depletion.

Non-ionic detergent extraction of cell membranes in cold conditions has been used to demonstrate the existence of detergent-resistant membrane domains or lipid rafts (Brown and London 1998). To further support our premise that rHIgM12 associates with membrane microdomains, the localization of human IgM was analyzed in cortical neurons treated with rHIgM12 and lysed at 4°C. Western blot analysis of the neuronal lysates with anti-human secondary IgM antibodies revealed membrane-bound rHIgM12 in both the pellet and the supernatant, whereas the non-neuron-binding control human IgM was found in only the wash eluates (Fig. 4a). Thus, rHIgM12 segregated into two pools, the 'detergent-soluble' one in the supernatant and the 'detergent-insoluble' one associated with the pellet. In a second study, cortical neurons were first treated with rHIgM12 in the cold followed by sucrose density gradient fractionation at 4°C. Western blot analysis of fractions indicated that rHIgM12 localized to the lighter fractions, which also contained caveolin-1, a lipid raft marker, but not to the transferrin receptor-enriched non-raft fraction (Fig. 4b). Even after this fractionation process (1% Triton X-100 and ultracentrifugation at 2×10^5 *g*), some rHIgM12 was detected in the pellet, which contains detergent-insoluble cytoskeletons (Fig. 4b). Although tubulin (Sorice *et al.* 2009) and actin (Levitan and Gooch 2007) have been found associated with lipid rafts, we detected only β3-tubulin in the raft fraction and the majority of actin in the non-raft fraction (Fig. 4b). This raft isolation method also indicated the two pools of membrane-bound rHIgM12 – one associated with lipid rafts and another with the pellet.

The antigen bound by rHIgM12 remains unknown. sHIgM12 binding was sialidase sensitive on primary cerebellar granule neurons (Warrington *et al.* 2004). rHIgM12 binding to neurons was unaffected by EDO-P4, which blocks glucosylceramide formation (Dubois *et al.* 1990; Lee *et al.* 1999) (Figure S1a and b) or fumonisin B1, which inhibits ceramide synthesis (Wu *et al.* 1995; Warrington *et al.* 2004). However, rHIgM12 binding to neuronal membranes was substantially decreased upon trypsin treatment (Figure S1a and c), although rHIgM12 did not bind to any discrete bands when tested on western blots with lysates from brain tissue or neurons (data not shown).

rHIgM12 modulates microtubule dynamics

rHIgM12 localized to the neuronal cell body, neurite shaft and growth cone central domain (Fig. 1), regions dominated by microtubules (Forscher and Smith 1988) and was detected in lipid raft fractions along with tubulin. To determine whether the rHIgM12 antigen is associated with the cytoskeleton through a link in membrane microdomains, hippocampal neurons were treated with rHIgM12 at 37°C to allow membrane rearrangement; then cells were simultaneously fixed and extracted at 37°C with 4% paraformaldehyde containing 0.1% Triton X-100 (Fig. 5a). After this procedure, rHIgM12 was localized to detergent-insoluble membrane *puncta* aligned with bundled microtubule fascicles in the neurite shaft (Fig. 5a1, a2 and a4). At the growth cone, rHIgM12 localized to the central domain, which is populated by defasciculated microtubules but did not bind to the F-actin-enriched growth cone periphery (Fig. 5a1, a3 and a4). The specific localization of rHIgM12 suggested that

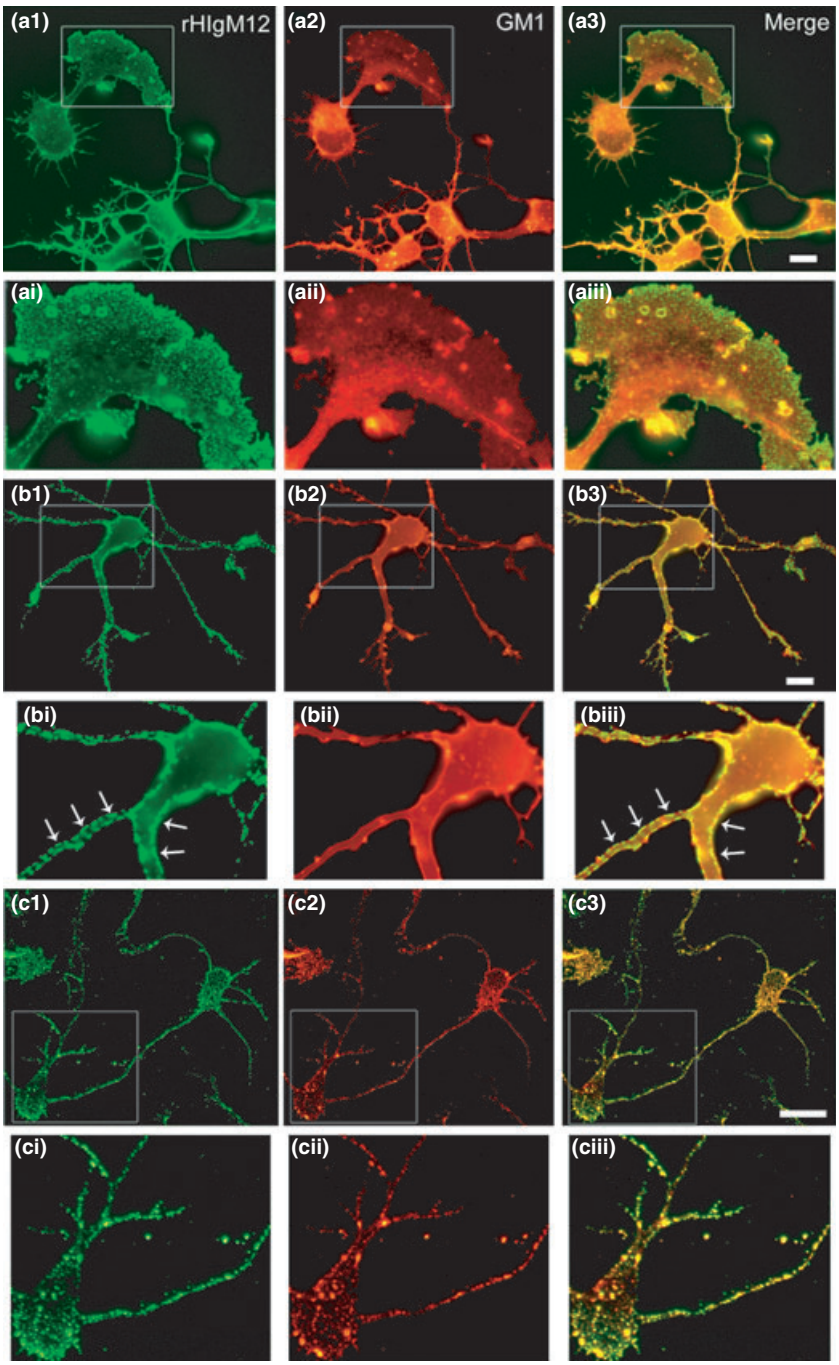

Fig. 3 rHIgM12 co-localizes with GM1 following cholesterol depletion. (a) DIV1 hippocampal neurons were fixed with 4% paraformaldehyde at 37°C first and then double stained by rHIgM12 and CTB. rHIgM12 localized to smaller punctate structures (green), which were partially co-localized with CTB-stained GM1 (red). (b) Live DIV1 hippocampal neurons were cooled down to 4°C and then double labeled with rHIgM12 and CTB. rHIgM12 localized to much larger punctate structures (green) on neuronal membranes (arrows), whereas GM1 (red) was relative evenly distributed. (c) Live DIV3 hippocampal neurons were treated with methyl-β-cyclodextrin for 30 min at 37°C to deplete cholesterol and then cooled down to 4°C and double stained with rHIgM12 and CTB. rHIgM12 (green) bound to the *puncta*, which were co-localized with GM1 (red). The lower panel [a(i–iii), b(i–iii) & c(i–iii)] showed higher magnification of the boxed regions. Scale bar = 10 μm.

rHIgM12-bound membrane molecules may function as microtubule anchors. To investigate whether membrane-anchored tubulin is coupled to signaling molecules that span the outer leaflet of the neuronal membrane, a modified pull-down experiment was performed at 4°C using neuronal lysates isolated from rHIgM12-treated cortical neurons. We

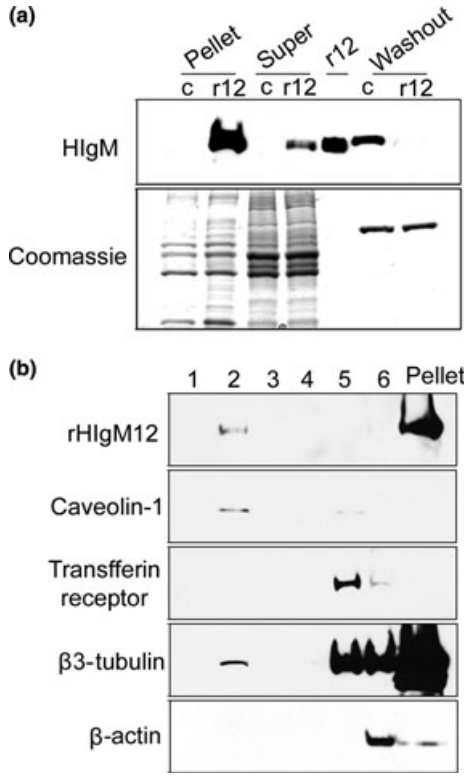

Fig. 4 rHIgM12 binds to lipid rafts. (a) Live DIV7 cortical neurons were treated with rHIgM12 (r12) or control IgM (c) antibody at 4°C for 30 min. Neurons were washed three times after antibody binding and lysed in buffer containing 0.5% NP-40. The lysates were separated by micro-centrifugation (1.61×10^4 *g*) at 4°C, and both the supernatant (Super) and pellet blotted with anti-human IgM secondary antibody. rHIgM12 (r12) distributed into both the pellet and the supernatant. In contrast, the control human IgM antibody (c), which did not bind to neurons, went to the washout. The lower panel showed a separate Coomassie-stained gel loaded with the same amount of protein. (b) Neurons lysed in buffer containing 1% Triton X-100 were fractionated by ultracentrifugation (2×10^5 *g*) in sucrose gradients at 4°C. The resulting fractions were sequentially blotted for rHIgM12, caveolin-1, transferrin receptor, β3-tubulin and β-actin with specific antibodies. rHIgM12 localized to the low-density fraction, which contained caveolin-1 and β3-tubulin. The higher density fractions contained transferrin receptor, β3-tubulin and β-actin. Some rHIgM12 localized to the detergent-insoluble pellet, which was mainly composed of cytoskeletal proteins enriched with tubulin. The majority of β-actin went to detergent-soluble higher density fractions.

found that rHIgM12 and β3-tubulin co-immunoprecipitated with each other (Fig. 5b), suggesting that molecules bound by rHIgM12 and β3-tubulin exist as a complex. These results further support the hypothesis that both tubulin and rHIgM12-bound molecules are associated in the same membrane microdomains.

To rule out the possibility that rHIgM12 binds to tubulin or microtubules non-specifically in neuronal lysates, pull-down assays were carried out using N2A neuroblastoma cells, which are not recognized by rHIgM12 but do express β3-tubulin. β3-tubulin was neither pulled down by rHIgM12 nor was IgM present in the pellet – it was detected only in the supernatants of N2A neuroblastoma cell lysates (Figure S2). Along with β3-tubulin, a small amount of actin was pulled down by rHIgM12 from cortical neurons (Figure S3a), suggesting that rHIgM12 bound to a complex containing both actin and β3-tubulin. However, rHIgM12-labeled *puncta* remained in the outer edge of the growth cone, whereas F-actin networks receded from the growth cone periphery when the hippocampal neurons were treated with rHIgM12 at 4°C (Figure S3b). After sucrose gradient fractionation of cortical neurons, actin localized to the non-raft fraction, and only a small amount of actin was detected in the detergent-insoluble pellet (Fig. 4b). These results suggest that F-actin was dynamic, and the majority was depolymerized at cold conditions and/or by detergent extraction.

To determine whether rHIgM12 binding to the surface of neurons modulated microtubule dynamics, DIV3 cortical neurons were treated with rHIgM12 for different lengths of time and cell lysates analyzed by western blot using antibodies against tyrosinated and acetylated α-tubulin. Tyrosinated α-tubulin was substantially increased after 30 min of rHIgM12 treatment, and the elevation was sustained as long as 2 h. In contrast, acetylated α-tubulin increased only slightly, and total α-tubulin did not change (Fig. 5c). More tyrosinated α-tubulin was synthesized in the presence of rHIgM12 indicating the IgM-induced modification of the neuronal cytoskeletons.

rHIgM12 promoted axon outgrowth

The typical feature of neurites that become axons is the competitively faster extension rates accompanied by accumulation of signaling molecules in the outer segment. sHIgM12 promotes neurite outgrowth in primary cerebellar granule neurons (Warrington *et al.* 2004). However, the early stage cerebellar granule neurons bore only one or two neurites when differentiating *in vitro*. The morphologically simple granule cells yielded limited information about human IgM-regulated neuron differentiation.

To confirm that rHIgM12 induced neurite extension in neurons, neurite outgrowth assays were conducted using primary hippocampal neurons. When presented as a substrate, rHIgM12 substantially promoted hippocampal neuron differentiation (Fig. 6). Within 12 h hippocampal neurons growing on rHIgM12 developed multiple neurites, one of them much longer than the neighboring neurites. In contrast, neurons seeded onto substrates of control human IgMs were mostly unattached. Those few attached neurons extended one or two short neurites (data not shown). Neurons plated on PDL developed multiple symmetric neurites. Neurite lengths from neurons plated on rHIgM12 ($n = 86$), when compared with the lengths of those on PDL ($n = 74$), were significantly longer (195.8 μm vs. 150.7 μm, $p = 0.0056$), the longest

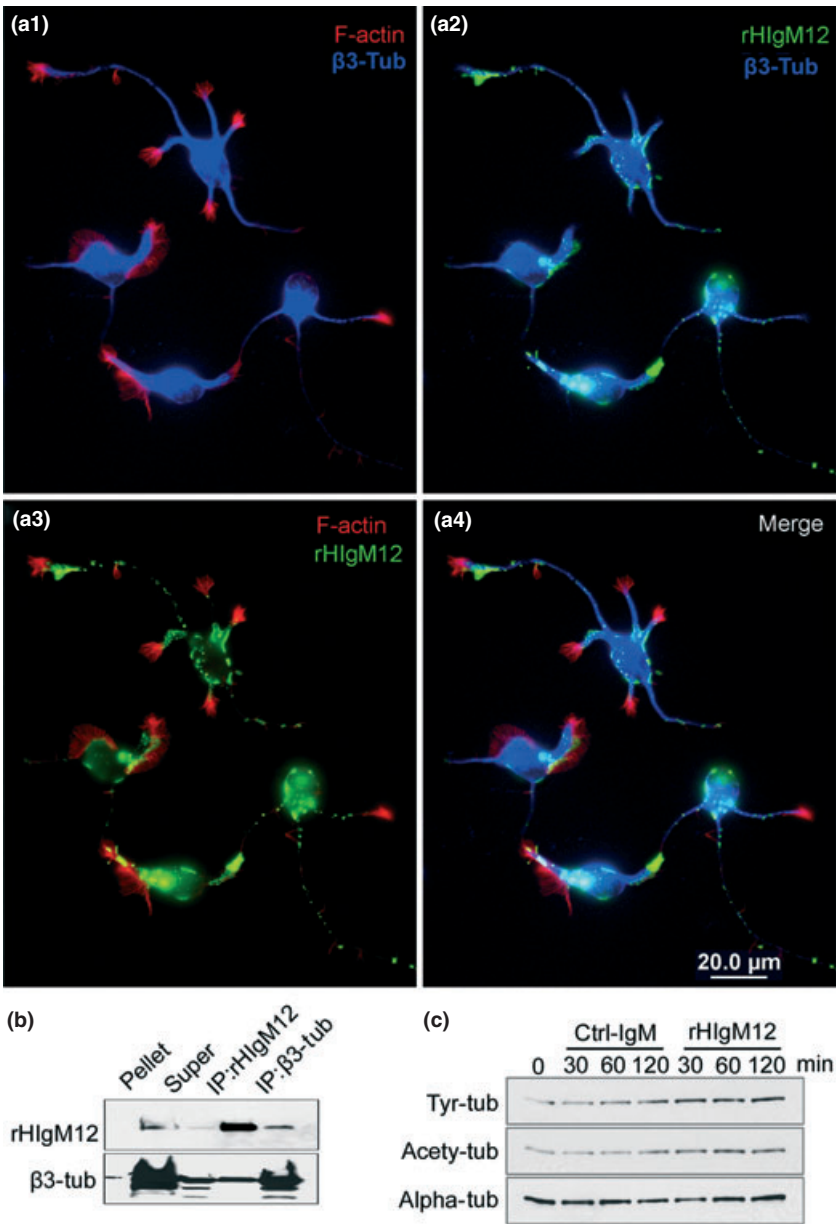

Fig. 5 rHIgM12 regulates microtubule dynamics. (a) DIV1 hippocampal neurons were treated with rHIgM12 for 30 min at 37°C, then simultaneously fixed and extracted with buffer containing 4% paraformaldehyde and 0.1% Triton X-100 and followed by immuno-staining to show rHIgM12 (green), microtubule (blue) and F-actin (red). rHIgM12 bound to the detergent-insoluble molecules, which co-localized with the fasciculated microtubules along the neurite shafts (a1, a2 & a4) and the growth cone central domain, but not with F-actin, which was mainly enriched in the growth-cone periphery (a1, a3 & a4). (b) DIV7 cortical neurons were allowed to bind rHIgM12 at 4°C for 30 min and then lysed in buffer containing 0.5% NP-40. The supernatants (Super) were subjected to co-immunoprecipitation with β3-tubulin. Both rHIgM12 and β3-tubulin (β3-Tub) were co-immunoprecipitated with each other. (c) DIV3 cortical neurons were treated with rHIgM12 at 37°C in the indicated time course. The total neuronal lysates were probed with anti-tyrosinated and acetylated α-tubulin (Tyr-Tub & Acety-Tub) antibodies. rHIgM12 substantially promoted tyrosination of α-tubulin. The result showed one of the three repeats. Scale bar = 20 μm.

neurite length was more than double the longest measured on PDL (117.1 μm vs. 51.8 μm, $p < 0.0001$). No difference in the second longest neurite length (38.7 μm vs. 33.3 μm, $p = 0.0782$) was found. Neurons growing on rHIgM12 bore fewer primary neurites (2.9 vs. 4.1, $p < 0.0001$) and the majority of them were of the stage-3 phenotype (72% on rHIgM12 vs. 18% on PDL). This piece of data show that not only does rHIgM12 promote neurite extension when presented as a substrate but also the IgM substantially promotes axonal differentiation (Fig. 6).

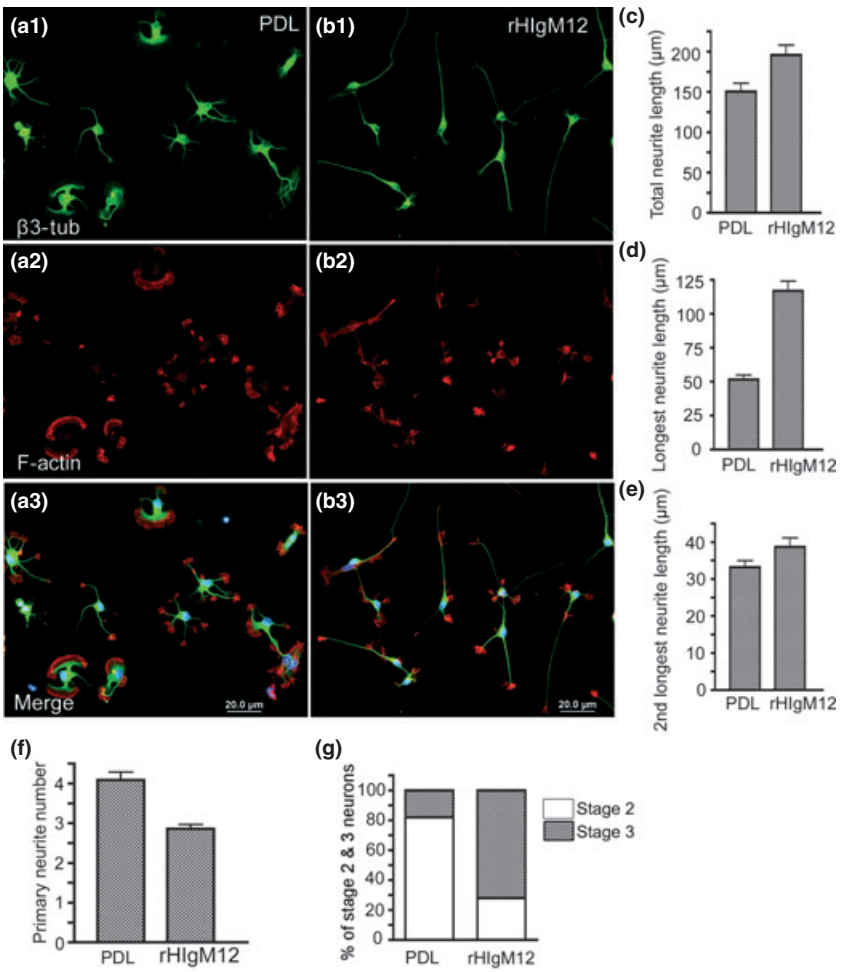

Fig. 6 rHIgM12 promotes neuron differentiation. (a–b) E15 hippocampal neurons were seeded on PDL (a) or rHIgM12 (b)-coated nitrocellulose glass coverslips. The neurons were fixed 12 h after plating and stained with β3-tubulin (β3-Tub) antibody (a1 & b1, green) and Texas-red phalloidin (a2 & b2). (a3–b3) are overlays of (a1–a2) and (b1–b2), where the nuclei stained with DAPI (blue). c, d & e show measurement of the total (c, 195.8 μm vs. 150.7 μm, $p = 0.0056$), longest (d, 117.1 μm vs. 51.8 μm, $p < 0.0001$) and 2nd longest (e, 38.7 μm vs. 33.3 μm, $p = 0.0782$) neurite lengths. Neurons growing on rHIgM12 bore fewer neurites (f, 2.9 vs. 4.1, $p < 0.0001$), and the majority were stage-3 neurons (g) as compared with those seeded on PDL (72% vs. 18%), that had multiple symmetric neurites. Statistical analyses show the mean ± SEM (unpaired *t*-test). Scale bar = 20 μm.

To verify that rHIgM12 facilitated axon extension, neurons growing on different substrates were stained with anti-*tau*1 or anti-MAP2 antibodies which label axons and dendrites, respectively (Fig. 7). *Tau*1 staining was quite weak in neurons grown on PDL but much stronger in neurons grown on laminin plus PDL or rHIgM12. Laminin is a classical substrate for neuron differentiation and axon formation (Chen *et al.* 2009). We used 50 μg/mL of laminin in PDL solution as a positive control, which is much higher than what is commonly used for neuronal cultures (4 μg/mL). Comparing the *tau*1 intensity in neurons grown on the various substrates, we found the distribution of *tau*1 to be asymmetrically enriched in the distal segments of the longest neurite and much higher in neurons plated on PDL-laminin or rHIgM12 (Fig. 7). MAP2 staining was found in the proximal portion of all neurites indicating that the longest neurites from the stage-3 neurons grown on either rHIgM12 or laminin had characteristics of axons (Fig. 7).

Discussion

One role of lipid rafts is to act as platforms that couple signaling from the membrane to the cytoskeleton (Fig. 8a). Lipids and proteins are continually incorporated into cell membranes, which may be then partitioned into microdomains. Membrane rafts are generally defined as nano scale (10–200 nm), heterogeneous, and dynamic membrane compartments enriched with sterols and sphingolipids (Lingwood

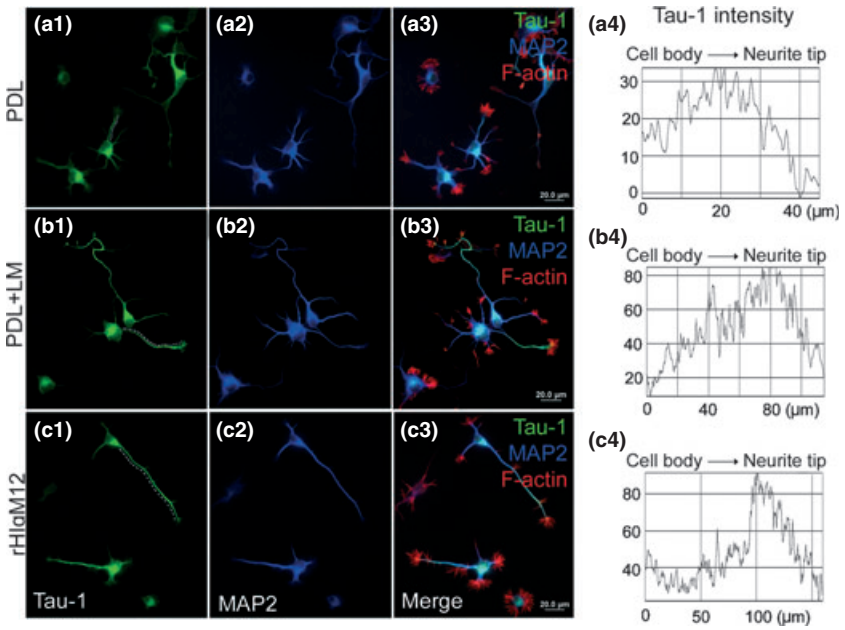

Fig. 7 rHIgM12 drives axon polarization. Hippocampal neurons 12 h after plating on PDL (a), PDL+Laminin (b) or rHIgM12 (c) were stained with *Tau*1 (a1–c1, green) or MAP2 (a2–c2, blue) antibody. (a3–c3) are overlays of (a1–c1) and (a2–c2), and F-actin (red) was labeled with Texas-red Phalloidin. (a4–c4) show the relative *Tau*1 intensity along the longest neurites from cell body to the growth cone in (a1–c1) labeled by dashed lines. *Tau*1 was asymmetrically enriched in the distal part of the longest neurite in stage-3 neurons grown on both rHIgM12 and laminin substrates. Scale bar = 20 μm.

and Simons 2010). In this study, we show that the neuron-binding recombinant human IgM, rHIgM12, binds to lipid rafts.

We observed that aggregates of cell surface-bound rHIgM12 co-localized with clustered cholesterol and ganglioside GM1. Following sucrose fractionation of lysates from neurons with membrane-bound rHIgM12, a pool of rHIgM12 localized into the lighter fractions, which contained the lipid raft marker, caveolin-1. rHIgM12 localized in aggregates of much larger *puncta* on neuronal membranes when hippocampal neurons were cooled to 4°C. This is consistent with the fact that cholesterol and sphingolipids have high Tm than other components of the membrane. Maintaining neurons below this Tm decreases lipid raft kinetics and facilitates the visualization of aggregated lipid rafts and/or associated molecules. rHIgM12 co-localized with GM1 after cholesterol depletion, suggesting that cholesterol modulates the kinetics of both rHIgM12-bound molecules and GM1. Smaller membrane rafts can interact with lipids and/or proteins. Individual tiny rafts can stabilize and fuse into larger platforms that integrate signals and regulate the intensity and amplitude of signaling pathways. IgM antibodies, which possess a pentameric structure that can bind up to 10 individual antigens, may impart signals to cells by cross-linking adjacent antigens (receptors) and/or bringing the antigens close enough to enhance cross-linking or interaction. As rHIgM12 was associated with microtubules, the antigen of rHIgM12 in the rafts likely mediated IgM-induced modulation of microtubules. The membrane raft fraction that contained rHIgM12 also contained β3-tubulin, and rHIgM12 and β3-tubulin co-immunoprecipitated with each other. Given the observation that tubulin can be cross-linked to membrane GM1 in neurons (Palestini *et al.* 2000), microtubules can apparently anchor to raft microdomains. The rHIgM12-membrane interaction may have illustrated a dynamic lipid platform, anchored by tubulin, through which membrane signaling is conveyed to microtubules (Fig. 8b).

We also demonstrated that rHIgM12, when used as a substrate, selectively promoted axon outgrowth from primary hippocampal neurons. F-actin and microtubules are the two major cytoskeletal elements involved in neurite extension and axon formation. F-actin, which localizes primarily to the growth cone periphery, was thought to play a major role in axon outgrowth; microtubules, which dominate the neurite shaft and are confined to the growth cone central domain, to be secondary to F-actin. However, microtubules have recently been shown to be critical in axon formation. Microtubules can interact dynamically with the growth cone actin meshwork, and microtubule stabilization can induce axon formation (Witte and Bradke 2008). Microtubules, composed of α and β-tubulin hetero-dimers, are dynamically regulated by extracellular cues during neurite extension. The finding that treatment with rHIgM12 increased tyrosination of α-tubulin further confirms the hypothesis that the asymmetric distribution of α-tubulin such that tyrosination occurs on the outside surface of microtubules, whereas acetylated

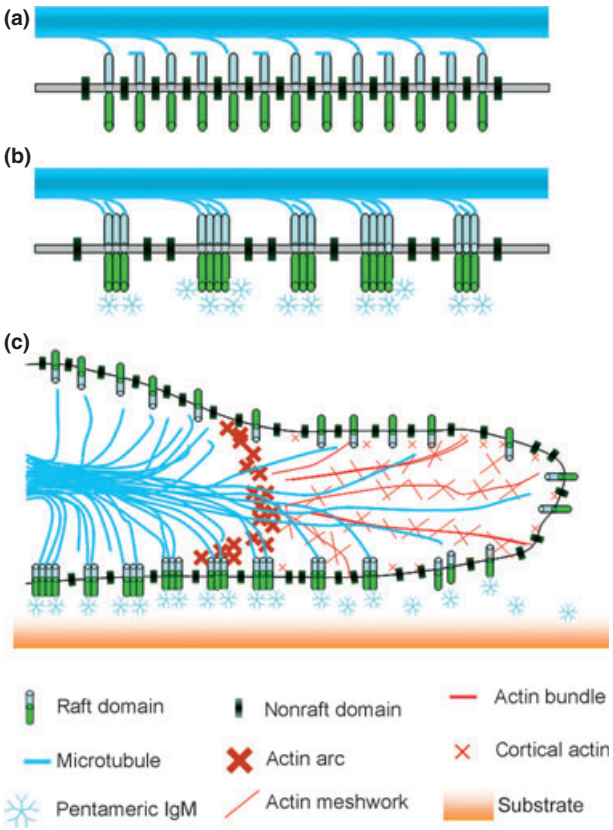

Fig. 8 Proposed mechanisms by which rHIgM12 promotes axon formation. (a) Neuronal membranes contain both raft and non-raft microdomains. The raft microdomains, which are evenly distributed on neuronal membranes, segregate into two pools. One of them is associated with microtubules. (b) Binding of rHIgM12 induces clustering of raft microdomains, which affects microtubule dynamics. (c) When presented as substrates, rHIgM12 interacts with and clusters raft microdomains. As a result, the microtubules are stabilized by anchoring to membrane domains, which may enhance growth cone periphery to central domain transition shown by the clustering of rHIgM12 within growth cone central domains (Figs 1 and 5).

residues point towards the lumen (Westermann and Weber 2003), is sufficient to mediate the process of axon formation.

Our results support microtubules as a central player in axon outgrowth and provide evidence of a direct microtubule interaction with the neuronal membranes. We have established that rHIgM12 and β3-tubulin co-immunoprecipitated with each other, that rHIgM12 co-localized with microtubules and that rHIgM12 was present in the pellet after detergent extraction of neuronal lysates. The ability of microtubules to grow and retract, for example in the growth cones, enables them to drive neuronal membrane movement (Dent and Kalil 2001), whereas stabilized microtubules can anchor neuronal membranes. The existence of dynamic and stabilized microtubules and the transition between these two states orchestrate the process of neurite outgrowth to specify axons (Figs 5, 6, 7 and 8c).

Presenting rHIgM12 as a substrate may immobilize and constrain the IgM's interaction with the neuronal membranes compared with rHIgM12 when added to the cell culture media. The immobilized rHIgM12 may create a gradient of signals across the neuronal membranes frequently similar to that observed in morphogen-induced signaling (Schmitt *et al.* 2006). Bath application of rHIgM12 may only facilitate random lipid raft clustering, without promoting asymmetric neurite outgrowth and enhancing axon formation. Our results using rHIgM12 confirm that neuronal membranes contain both raft and non-raft microdomains. There are two pools of raft domains, one of them associated with microtubules. We propose that rHIgM12 binds to and clusters lipid raft domains, which affects microtubule dynamics to drive axon extension. rHIgM12-induced raft clustering at the growth cone may be instrumental in enhancing the periphery to central domain transition that breaks symmetric neurite outgrowth to specify axon formation. Our data suggest that rHIgM12 binding recruits membrane molecules such as cholesterol and GM1 to IgM-neuronal membrane contact sites. This recruitment may activate down-stream signals required for axonal specification.

The ability of rHIgM12 to promote neurite and axon extension is reminiscent of other treatments, such as neuraminidase, ionophores, CTB and exogenous gangliosides that induce neurite extension from primary neurons and neuroblastoma cells (Wu *et al.* 1998). The common link among these treatments may be modulation of the level of organization of membrane glycosphingolipids, resulting in changes in raft content, allowing ion flux, clustering signaling molecules on the membrane and/or enhancing signal transduction. We propose that pentameric IgMs (Czajkowsky and Shao 2009) like rHIgM12 can act as signaling molecules in similar ways by clustering cell surface domains. CTB is also pentamer that can bind, with high affinity, five molecules of GM1 (Zhang *et al.* 1995). CTB promotes neurite extension and elevates intracellular Ca^{2+} in primary neurons (Milani *et al.* 1992; Wu *et al.* 1996). Treatments that elevate intracellular Ca^{2+} shifts the character of neurite extensions to axons (Wu *et al.* 1998). There is evidence that Ca^{2+} channels can be gated by altering their association with GM1 (Fang *et al.* 2002). These observations are quite intriguing because we have shown a significant correlation between the ability of multiple IgMs to promote remyelination and their ability to induce transient Ca^{2+} influx in the target cells, oligodendrocytes (Paz Soldan *et al.* 2003).

A study of extracellular laminin-induced neurite outgrowth (Ichikawa *et al.* 2009) has also proposed that reagents that drive the formation of microdomains can enhance signal transduction. These authors showed that laminin-mediated clustering of ganglioside GM1 shifts β1 integrin into lipid rafts where it more effectively interacts with TrkA. The resulting signal cascade activates Lyn, Akt, and MAPK resulting in neurite extension. We have also shown that a

recombinant human IgM that promotes remyelination induces Lyn activation in myelin forming cells (Watzlawik *et al.* 2010). The underlying principles of IgM signaling may be very similar in oligodendrocytes and neurons.

Exogenous gangliosides have been used as therapeutics for regeneration of the nervous system, for example after spinal cord injury (Geisler *et al.* 1991). A study by Rosen's group showed that treating cells with exogenous GM1 increases neurite extension by altering the distribution and interaction of actin and microtubules (Wang *et al.* 1998). However, long-term treatment with gangliosides is not feasible. The molecules are immunogenic, very insoluble and do not target the sites of injury. IgMs that induce axon extension may be a realistic alternative, as they are minimally antigenic to humans and have been shown to target to sites of nervous system damage (A. Warrington, unpublished observation).

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Patents for antibodies that promote remyelination and central nervous system repair are issued and are owned by Mayo Foundation. Therefore, the authors have a potential future financial conflict of interest.

Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1. rHIgM12 binding to neuronal membranes is sensitive to trypsin treatment.

Figure S2. rHIgM12 does not associate with tubulin directly.

Figure S3. rHIgM12 pulls down actin, but does not co-localize with bundled F-actin.

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