Phosphorylation of adhesion- and growth-regulatory human galectin-3 leads to the induction of axonal branching by local membrane L1 and ERM redistribution

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Summary

Serine phosphorylation of the β-galactoside-binding protein galectin-3 (Gal-3) impacts nuclear localization but has unknown consequences for extracellular activities. Herein, we reveal that the phosphorylated form of galectin-3 (pGal-3), adsorbed to substratum surfaces or to heparan sulphate proteoglycans, is instrumental in promoting axon branching in cultured hippocampal neurons by local actin destabilization. pGal-3 interacts with neural cell adhesion molecule L1, and enhances L1 association with Thy-1-rich membrane microdomains. Concomitantly, membrane-actin linker proteins ezrin-radixin-moesin (ERM) are recruited to the same membrane site via interaction with the intracellular domain of L1. We propose that the local regulation of the L1-ERM-actin pathway, at the level of the plasma membrane, underlies pGal-3-induced axon branching, and that galectin phosphorylation in situ could act as a molecular switch for the axon response to Gal-3.

Key words: Axon branching, Glycoprotein, Heparan sulphate, L1-ezrin-moesin-radixin-actin, Lectin, Plasma membrane

Introduction

The formation of the central nervous system (CNS) network requires active axonal elongation and a systematic exploratory activity of the environment by the growing axons in order to be directed to their correct targets. One of the neuronal strategies to sense the surroundings is the formation of branches, with growth cones responding to molecular information from the extracellular milieu that instructs the neuron in the process of maturation. Apart from the obvious importance of elongation and branching during CNS development, their regulation in the adult CNS would be an attractive tool to induce nerve regeneration in pathological situations such as traumas or degenerative diseases. Several glycoproteins play a decisive role in this process, among them the neural cell adhesion molecule L1 (Cheng and Lemmon, 2004; Itoh et al., 2004; Ledeen and Wu, 2009). In mature neurons L1 is sorted to the axonal compartment, and regulates axon elongation and branching by homophilic interactions that recruit the membrane-actin linkers ezrin-radixin-moesin (ERM) (Cheng et al., 2005). Within the membrane, L1 can associate with detergent-resistant membrane fractions (DRM or rafts), and the extension of this association influences its effects, switching between promotion and inhibition of neurite outgrowth (Kleene et al., 2001). In addition to homophilic recognition, the glycan chains of neuronal glycoproteins are also important as ligands for a lectin-mediated information transfer, embodied in the concept of the sugar code (Gabius, 2009). In fact, endogenous lectins are able to decode the information in the form of distinct glycan determinants to regulate cell adhesion, growth, migration and differentiation, the members of the galectin family being one such example (Gabius et al., 2004; Villalobo et al., 2006). These proteins target branch-end epitopes of glycan chains, acting as sugar-encoded switches from proliferation to anoikis or differentiation in cellular communication, and as attachment sites for adhesion (André et al., 1999; Kopitz et al., 2001). Owing to its trimodular design the chimera-type galectin-3 (Gal-3) is unique within this family. In addition to its carbohydrate recognition domain, it harbours a collagenase-sensitive stalk, instrumental for pentamerization in the presence of multivalent ligands (Ahmad et al., 2004), and an N-terminal sequence with sites for casein-kinase-1-dependent serine phosphorylation (Cowles et al., 1990; Huflejt et al., 1993; Kübler et al., 2008).

This post-translational modification has relevance, at the intracellular level, for nuclear export and anti-apoptotic activity (Takenaka et al., 2004; Yoshii et al., 2002). In order to further probe into its functional relevance we previously developed an efficient strategy to produce phosphorylated Gal-3 (pGal-3) in large quantities (Kübler et al., 2008). This procedure has made it possible to monitor carbohydrate and cell binding comparatively (Kübler et al., 2008; Szabo et al., 2009) and is a prerequisite for detailed functional studies. Having also previously initiated the monitoring of the involvement of galectins in axonal development with two prototype chicken galectins (Kopitz et al., 2004), the access to pGal-3 allows us to address the issue of the functional relevance of Gal-3 phosphorylation in this respect. Of note, Gal-3 is expressed mainly by glial cells (Pesheva et al., 1998; Reichert et al., 1994; Walther et al., 2000) but also by subsets of rat sensory and mouse dorsal root ganglia (DRG) neurons (Pesheva et al., 2000; Regan et al.,

1986). It promotes neural cell adhesion and neurite growth of DRG neurons (Pesheva et al., 1998) and, when covalently cross-linked by transglutaminase activity, it can stabilize the neurites of cerebellar granule cells (Mahoney et al., 2000). In this context, it is attractive to suggest that phosphorylation can act as a molecular switch, for instance, by masking an important site on Gal-3 or creating a new site, thereby modulating the activity profile of Gal-3.

In this study we first compared the bioactivity of Gal-3 and pGal-3 in the development of CNS neurons, using primary rat hippocampal and cortical cultures. Owing to its physiological production in situ we also tested the product of matrix-metalloproteinase-dependent Gal-3 proteolysis that is the truncated (trGal-3) as it lacks the N-terminal portion with phosphorylation site(s) and the central collagenase-sensitive stalk region. We show that adsorbed, but not soluble, pGal-3 targets L1, induces its redistribution to Thy-1-containing rafts and, upon recruitment of the membrane-actin linkers ERM, stimulates local axon branching.

Results

Adsorbed phosphorylated Gal-3 induces axon branching

The first parameter tested in vitro was axon growth. To examine the spectrum of effects of Gal-3, each naturally occurring form of this lectin was added to the neurons in solution or adsorbed to the surface of the coverslips. Neither the full-length protein without phosphorylation, the proteolytically truncated (trGal-3) nor the phosphorylated variants triggered a response. Quantitatively, treated neurons grew axons that were, on average, no more than 20 μm longer than the control ones (Fig. 1A,B; solution, substratum). However, of particular note, neurons cultured on pGal-3-covered surfaces revealed a novel activity of pGal-3: causing a marked increase in neurite branching (Fig. 1A, regions 3 and 4). Control neurons and those grown on Gal-3 or trGal-3 showed an average branching level of their longest neurite between 1 and 2, whereas for neurons on pGal-3 this parameter peaks at 4-5 (Fig. 1C). This effect was not sensitive to the presence of 25 mM of the haptenic inhibitor lactose, a concentration well tolerated by cultured hippocampal neurons (Fig. 1C).

The fact that pGal-3 only triggered axon branching if immobilized is indicative of how pGal-3 is presented to the neuronal membrane. A similar effect might thus be triggered by presentation via components of the extracellular matrix (ECM). To test this assumption, complexes of different forms of Gal-3 with heparan sulphate proteoglycans (HS) were used. HS were selected in preference to other available ECM molecules because of their lack of intrinsic influence on axon development; other ECM components either elicit axon growth (laminin or fibronectin) or actively inhibit it (chondroitin sulphate proteoglycans). The pGal-3–HS complexes (ratio 5:1, w/w), added to neuron cultures for 24 hours, also

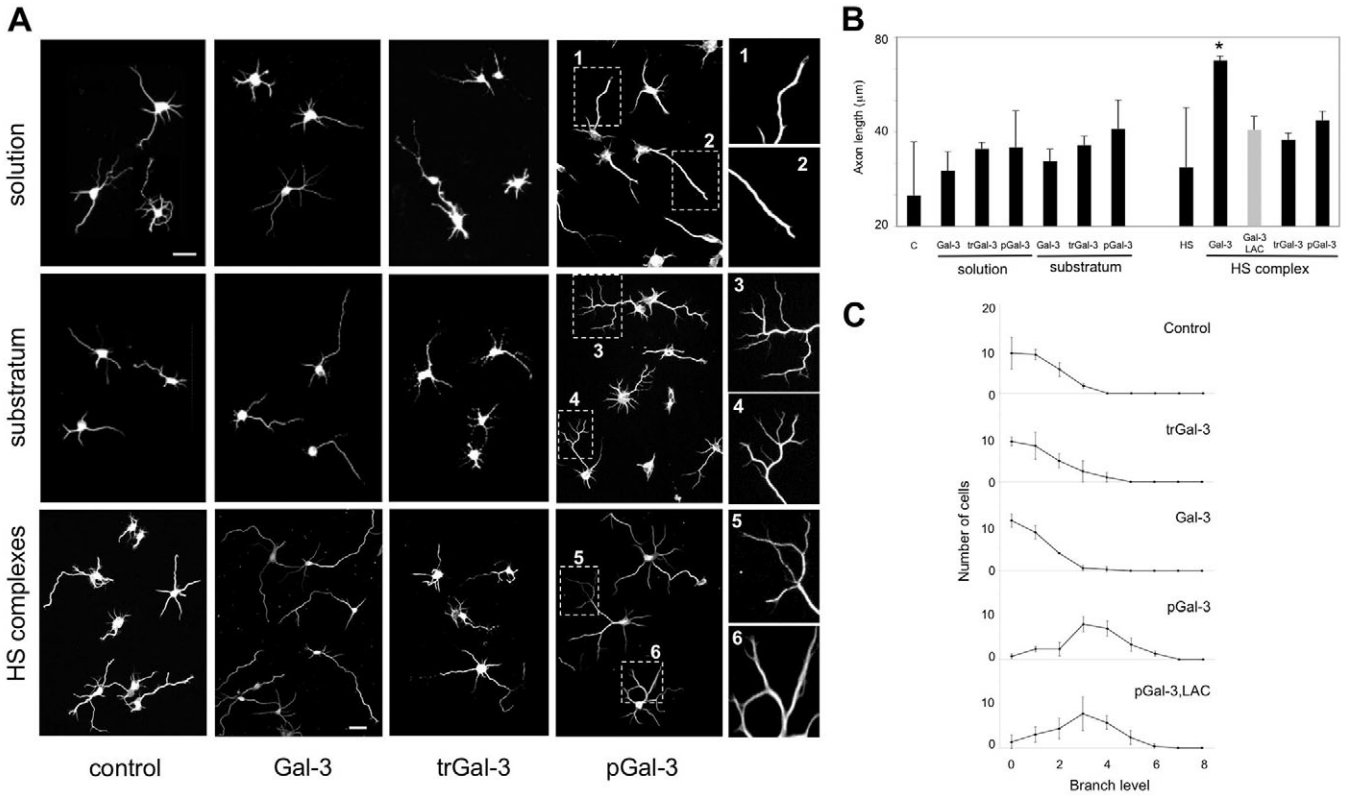

Fig. 1. Bound pGal-3 induces axon branching. Hippocampal neurons were treated for 48 hours with different forms of human Gal-3 either in solution (25 μg/ml), covering the surface of the coverslips (substratum), or as soluble complexes with heparan sulphate proteoglycans (HS; 25 μg/ml, final galectin concentration; galectin–HS, 5:1). Controls were similar cultures on PLL-covered surfaces, or treated with the same HS concentration. (**A**) Neurons fixed and immunolabeled with anti-α-tubulin antibodies. The enlarged regions 3-6 show details of highly branched axons. (**B**) Bar graph of the effect on axon length of different Gal-3 forms. The grey bar shows the effect on Gal-3–HS-treated neurons in the presence of lactose. Values are means + s.d. of three experiments (*P<0.001). Axon length was measured using ImageJ software. (**C**) The effect of different Gal-3 forms on branching in populations of neurons (axon = level 0; branch from axon = level 1; branch from level 1 = level 2, etc.). Branches were counted manually. Experiments were performed in the presence of 25 mM lactose where indicated (LAC). Values are means ± s.d. of three experiments (25 neurons per experiment). Scale bars: 10 μm.

established the branched phenotype (Fig. 1A, regions 5 and 6), as generated by pGal-3 present on the substratum. This activity suggests that these pGal-3-HS complexes may associate preferentially with branching nodes in distal axon domains. In fact, living neurons, incubated with the pGal-3–HS complexes prepared with biotinylated lectin, mainly label branching points of distal axon tracts (Fig. 2A, arrows; Fig. 2C). pGal-3 alone, used as control, did not bind to nodes and showed low and diffuse labelling underscoring the impact of mode of presentation (Fig. 2B, arrows; Fig. 2C).

In agreement with previous reports using plastic-adsorbed Gal-3 and cell lines (Pesheva et al., 1998), Gal-3–HS complexes under the same conditions substantially induce axon growth (Fig. 1A). Gal-3–HS-stimulated axons are, on average, 35 μm longer than controls (Fig. 1B). The lectin activity can be neutralized by the presence of 25 mM lactose, revealing a high level of sensitivity to the lectin-blocking sugar (Fig. 1B, grey bar).

Axon branching and guidance share several regulatory molecules (Yoshida et al., 2008) such as semaphorins, TAG-1 (Liu and Halloran, 2005) or STAT3 (Conway, 2006). Additionally, prototype galectins (galectin-1 and related family members) are involved in axonal guidance in vitro (Kopitz et al., 2004) and in vivo (Gaudet et al., 2005; Puche et al., 1996). This prompted us to explore whether the effect of pGal-3 may not be specific to branching and might modulate axon guidance as well. To address this question we tested whether unmodified Gal-3 or its phosphorylated and truncated forms are able to guide growing axons in vitro. To do so, axons were grown on galectin and PLL alternated substrata. Under these conditions, axons did not show any preference for growth along parallel stripes covered only with PLL (negative control; Fig. 3, FITC), and instead most of them tended to extend along laminin stripes (positive control; Fig. 3 laminin, black bar). In the case of stripes covered with any of the three forms of Gal-3, axons did not show any preference to extend along them or to be confined to PLL stripes (Fig. 3, Gal-3, trGal-3, pGal-3, black and light grey bars). In fact, axons tended to grow across trGal-3 or pGal-3 stripes ignoring any transition between PLL and galectin zones (Fig. 3, trGal-3, pGal-3, dark grey bars). These results suggest a possible role of Gal-3 in axon guidance, although pGal-3 modulation of branching activity seems to be specific, as it is not triggered by trGal-3.

Phosphorylated Gal-3 induces an abnormal number of F-actin-containing branches

The formation of a new branch requires local cytoskeleton remodelling to determine its location as well as to trigger and maintain its growth. To probe into the possibility for such a rearrangement of the cytoskeleton by pGal-3 in complexes with HS, hippocampal neurons were treated either with HS alone or with Gal-3–HS complexes, and immunolabelled for α-tubulin and F-actin. Most of the HS-treated neurons had a typical stage-3 phenotype (Dotti et al., 1988) with axons showing few microtubule-containing branches (Fig. 4A, arrows). These axons also had a few small protrusions enriched in F-actin (Fig. 4A, arrowheads). By contrast, treatment with pGal-3–HS induced a highly branched phenotype characterized by a large number of microtubule-containing branches (Fig. 4B, arrows) and extended zones showing abnormal profusion of F-actin-labelled branchlets (Fig. 4B, arrowheads; Fig. 4C).

Bound pGal-3 interacts with L1

In order to define targets of pGal-3 on the cell surface, neuron cultures were incubated with pGal-3–HS complexes at 12°C, to minimize endocytosis, using Gal-3–HS complexes as control. Binding partners were conjugated with a cross-linker forming

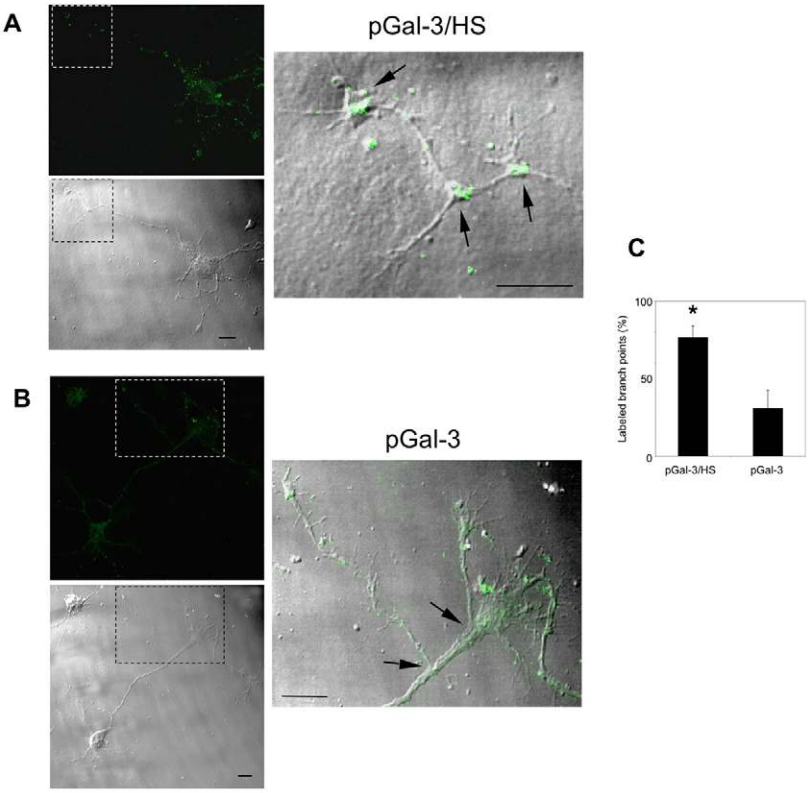

Fig. 2. pGal-3–HS complexes bind preferentially to branching points. (**A**,**B**) Hippocampal neurons were cultured for 72 hours on glass coverslips and incubated for 1 hour, at 12°C, in buffered medium with biotinylated pGal-3 (25 μg/ml), in complexes with HS (A), or alone (B). After washing, neurons were incubated with FITC-conjugated streptavidin in the same conditions. Cells were then fixed and mounted. The boxed areas are shown at higher magnification on the right and are DIC and fluorescence merged images. Arrows indicate branching points, labelled only after pGal-3–HS incubation. Scale bars: 10 μm. (**C**) The percentage of labelled branch points. We consider 'positive' those branch points showing three times higher fluorescence intensity than the background measured in adjacent neurite tracts. Values are mean ± s.d. of three experiments (20 fields per experiment, **P*<0.005).

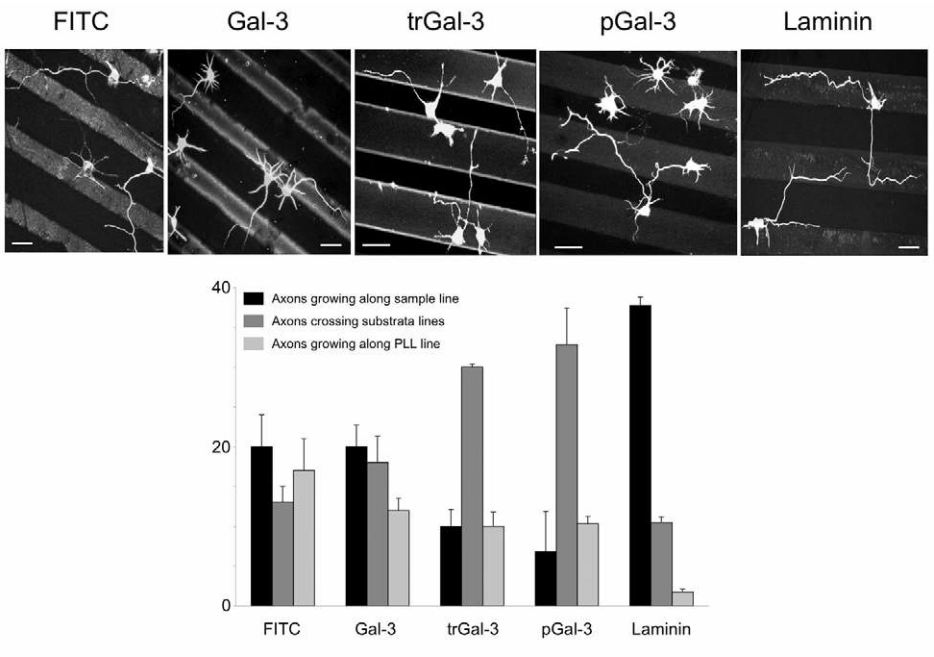

Fig. 3. Gal-3 does not affect axon guidance in vitro. Hippocampal neurons cultured for 48 hours on coverslips coated with parallel stripes of FITC together with the indicated form of galectin, FITC alone as negative control, or together with laminin as positive control (light stripes). Anti-tubulin staining was performed to visualize the cells. Neurons extend axons along laminin stripes (laminin) but they do not show any preference for any of the forms of Gal-3 (central) or FITC alone (left). The bar graph below gives the number of axons growing along each substratum (black bars), out of them (light grey bars) or across them (dark grey bars). Values are mean ± s.d. of three experiments (50 cells per experiment). Scale bars: 10 μm.

cleavable disulfide bridges between the two proteins. After immunoprecipitation with anti-Gal-3 antibodies, disulfide bridges were cleaved by 2-mercaptoethanol, present in the Laemmli buffer. Samples were then analyzed by western blotting with antibodies against β1-integrin, neuropilin-1 or L1, known as galectin ligands. Anti-Gal-3 antibodies immunoprecipitated 60% more L1 from neurons incubated with pGal-3 than with Gal-3, while β1-integrin and neuropilin-1 were not detected (Fig. 5A). To independently corroborate that L1 is a binding partner of pGal-3, samples from parallel cultures were immunoprecipitated with anti-L1 antibodies, after the same crosslinking procedure, and analyzed by western blotting with anti-Gal-3 antibodies. Consistently, the lectin was almost exclusively detected in immunoprecipitates from pGal-3-treated neurons (Fig. 5B). This coimmunoprecipitation of pGal-3 and L1 constitutes firm evidence for an interaction between the two molecules, and suggests that such an interaction might trigger the observed branching response.

Bound pGal-3 induces L1 localization to Thy-1-rich clusters in living neurons

The enhanced number of branches and the highly dynamic actin cytoskeleton observed upon exposure of neurons to adsorbed pGal-3 resembles the phenotype induced by forced L1 clustering (Cheng et al., 2005; Dickson et al., 2002). We consequently tested whether the interaction of adsorbed pGal-3 with the axon influenced L1 presentation within the membrane. To address this question, two possible scenarios were considered. First, L1 could form more and/or bigger clusters and, second, they could associate with different membrane sub-compartments, as L1 activity in axon membrane has been associated with membrane rafts (Nakai and Kamiguchi, 2002).

Using the co-patching technique on living cultured neurons (Abad-Rodriguez et al., 2004), L1 and Thy-1 were labelled with specific antibodies and patched with fluorophore-conjugated secondary antibodies. L1 clusters, revealed by this antibody patching, did not show any evident change in quantity or size upon pGal-3 interaction (Fig. 6A,B, L1). In fact, the percentages of cluster size families (<3, 3-12, >12 in pixels) were similar to those of control neurons (Fig. 6C). In order to test the second possibility, L1 and the raft marker Thy-1 were co-patched in the same experiments. As for L1, the Thy-1 cluster size was not affected by pGal-3 interaction (Fig. 6C), although neurons grown on pGal-3 showed an increased colocalization of L1 and Thy-1 clusters, when compared with controls (Fig. 6A,B). Quantitatively, in control cells around 50% of L1 clusters (Fig. 6D) colocalized with Thy-1, and non-colocalizing L1 clusters were present (Fig. 6A, merge, arrows). By contrast, pGal-3-treated neurons had around 70% colocalization (Fig. 6D). In this case, the colocalization is even more prominent at branching points (Fig. 6B, merge, arrowheads). As a consequence of this immunocytochemical approach, a higher proportion of L1 should be present in raft fractions after cells have been exposed to pGal-3–HS complexes. To test this biochemically, membranes from neurons cultured in the presence of Gal-3 or pGal-3 were extracted in cold 1% Triton X-100 solution, fractionated in sucrose gradients and analyzed by western blotting. Indeed, a higher quantity of L1 was detected in raft fractions of neurons treated with pGal-3, when compared with both controls (see later) or with neurons treated with Gal-3 (supplementary material Fig. S2A,B).

Bound pGal-3 induces ERM localization to Thy-1-rich clusters

L1-mediated branching involves interaction with the membrane-cytoskeleton linker proteins ERM (Cheng et al., 2005). The functional relevance of L1 redistribution would become apparent if ERMs were subject to similar redistribution. We thus tested whether these proteins are displaced to Thy-1-rich membrane microenvironments. Control neurons showed essentially segregated ERM and Thy-1 distributions in punctate patterns (Fig. 7A, merge, arrows). By contrast, adsorbed pGal-3-treated neurons had more-or-less continuous labelling, with accumulations of both proteins at the branching points (Fig. 7B), where a high level of colocalization was seen (Fig. 7B, merge, arrows; Fig. 7C). These results indicate that the specific membrane-cytoskeleton linkers ERM, necessary for L1-mediated branching, were recruited to the

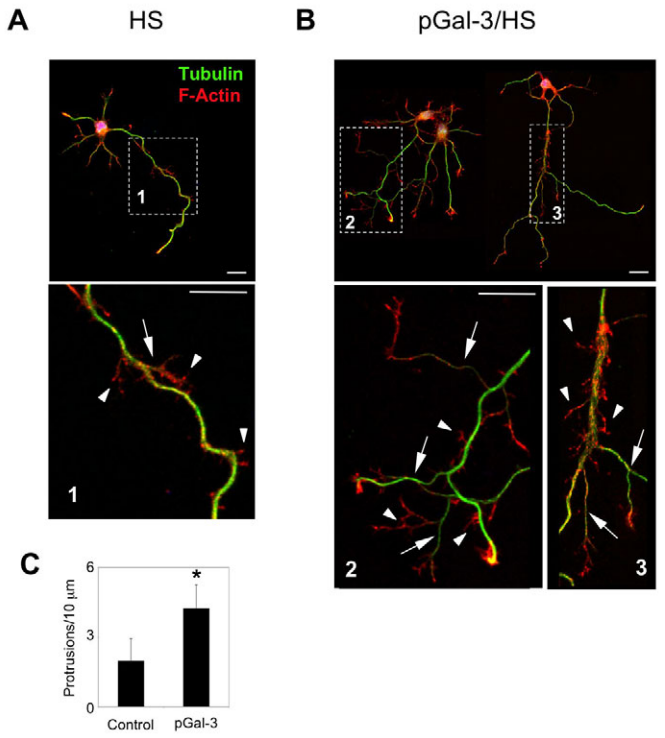

Fig. 4. Bound pGal-3 induces local changes in the F-actin cytoskeleton. (**A**,**B**) Hippocampal neurons were cultured for 72 hours in the presence of HS alone (A), or pGal-3–HS complexes (25 µg/ml; B). Cells were fixed and immunolabelled for α-tubulin (green) and for F-actin (red). pGal-3–HS-treated cells had more tubulin-containing branches (arrows) and a much higher number of F-actin-positive protrusions (arrowheads) than HS-treated neurons. Scale bars: 10 µm. (**C**) Number of F-actin-labelled protrusions per 10 µm of neurite length is represented. Protrusions were counted manually and neurite length was measured as in Fig. 1. Results are mean ± s.d. of three experiments (20 fields per experiment, **P*<0.005).

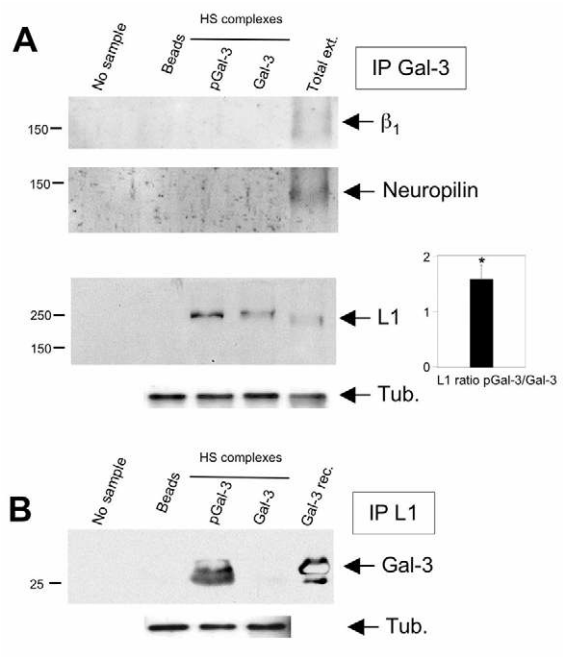

Fig. 5. Bound pGal-3 interacts with L1. Cortical neurons were cultured in plastic dishes for 7 days, and incubated for 1 hour, at 12°C, in Locke's solution, with Gal-3 or pGal-3 (25 µg/ml) in complex with HS. Cells were exposed to the crosslinking reagent DTSSP, quenched with Tris buffer (pH 7.5) and scraped into lysis buffer. After centrifugation, supernatants were immunoprecipitated with anti-Gal-3 antibodies and blotted for β1-integrin, neuropilin-1 and L1 (**A**). Around 60% more L1 was coimmunoprecipitated by pGal-3–HS compared with Gal-3–HS (bar graph; values are mean ± s.d. of three experiments, **P*<0.005). (**B**) Parallel immunoprecipitation with anti-L1 antibodies and blotting for Gal-3 confirmed L1–pGal-3 interaction. The same quantities of extracts were blotted for α-tubulin as loading controls.

same membrane domains (Thy-1-enriched rafts) upon axon interaction to bound pGal-3.

Biochemical membrane fractionation further supported these findings. As mentioned above for L1, ERM were detected in raft fractions of neurons grown on pGal-3, while they were only poorly associated with membranes of control neurons (Fig. 9F, central panel and graph), or of neurons grown on Gal-3 (supplementary material Fig. S2C).

Expression of a dominant-negative L1 form and perturbed raft formation impair pGal-3-induced branching

In order to directly test the involvement of membrane rafts and the role of L1 in branching induced by bound pGal-3, we tested the model under conditions that perturb either raft formation or L1 signalling.

In a first set of experiments we treated cultures with 500 mM methyl-β-cyclodextrin (MCD). This protocol leads to extraction of 25-30% of neuronal membrane cholesterol and perturbs the organization of rafts, as indicated by partial displacement of Thy-1 from the raft fractions (Fig. 9F, bottom panel and graph), in agreement with our previous report (Abad-Rodriguez et al., 2004). This lowering in membrane cholesterol results in the suppression of the pGal-3 effect on axon branching (Fig. 8A,C), indicating that physiological raft organization is necessary for induction of branching.

In the second set of experiments we expressed a dominant-negative form (DN) of L1 (L1-1151Y>A) in cultured neurons. It harbours a single mutation at the juxtamembrane zone of L1, which impairs L1-ERM binding and precludes axon branching triggered by L1-L1 homophilic interaction. This mutation does not affect axon growth (Cheng et al., 2005). Bound pGal-3-treated neurons expressing DN-L1 show an impaired induction of branching, with axons displaying few or no branches (Fig. 8B,C). These results further demonstrate the participation of L1-ERM signalling in the induction of branching triggered by bound pGal-3.

Bound pGal-3 induces colocalization of L1 and ERM

So far, we have shown that bound pGal-3 simultaneously displaces L1 and ERM to the Thy-1-enriched plasma membrane domains. In order to reveal interaction of both proteins within these domains we conducted colocalization experiments with L1 and ERM. Control neurons showed L1 and ERM in punctate patterns with low level of colocalization, even in branching points (Fig. 9A, merge, arrows; Fig. 9E). By contrast, bound pGal-3-treated neurons showed continuous labelling, more intense in branching points, with a marked level of colocalization of L1 and ERM (Fig. 9B, merge, arrows). The expression of the dominant negative form of L1 (Fig. 9C), or the perturbation of the membrane raft by partial cholesterol extraction with MCD (Fig. 9D), precluded the effect of bound pGal-3, reducing L1 and ERM colocalization down to control levels (Fig. 9E). These results indicate that, upon axon interaction with bound

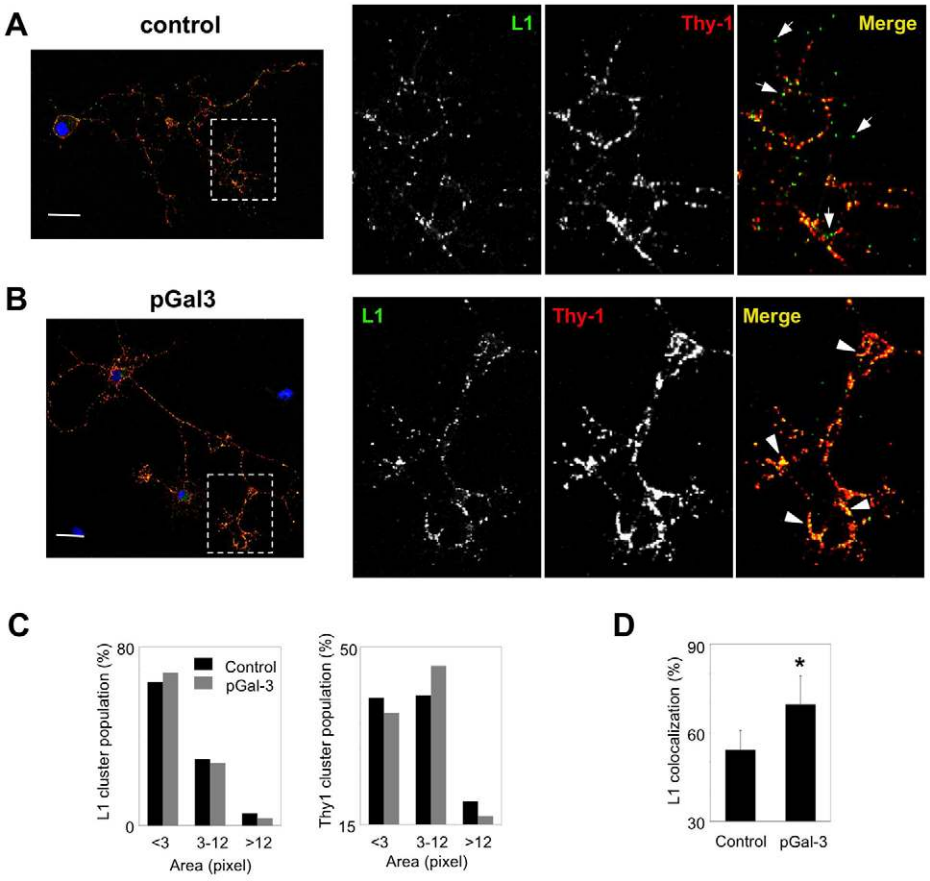

Fig. 6. Bound pGal-3 displaces L1 to Thy-1-containing membrane rafts. (**A**,**B**) Hippocampal neurons were cultured for 72 hours on PLL (A) or pGal-3-covered coverslips (B). Living neurons were incubated for 1 hour, at 12°C, in buffered medium, with anti-L1 and anti-Thy-1 antibodies. After washing, cells were patched with fluorophore-conjugated secondary antibodies under the same conditions, fixed and mounted. Arrowheads indicate co-patched clusters. Arrows indicate non-co-patched L1 clusters. L1 and Thy-1 clusters were classified into three families according to their size in pixels (<3, 3-12, >12). (**C**) Percentages of clusters of each family in control cultures and pGal-3-treated cultures No noticeable variation in cluster size was detected. (**D**) The percentage of L1 clusters co-patching with Thy-1 clusters is significantly higher in neurons cultured on pGal-3 than in controls. Results are mean ± s.d. of three experiments (20 fields per experiment; **P*<0.005). Scale bars: 10 µm.

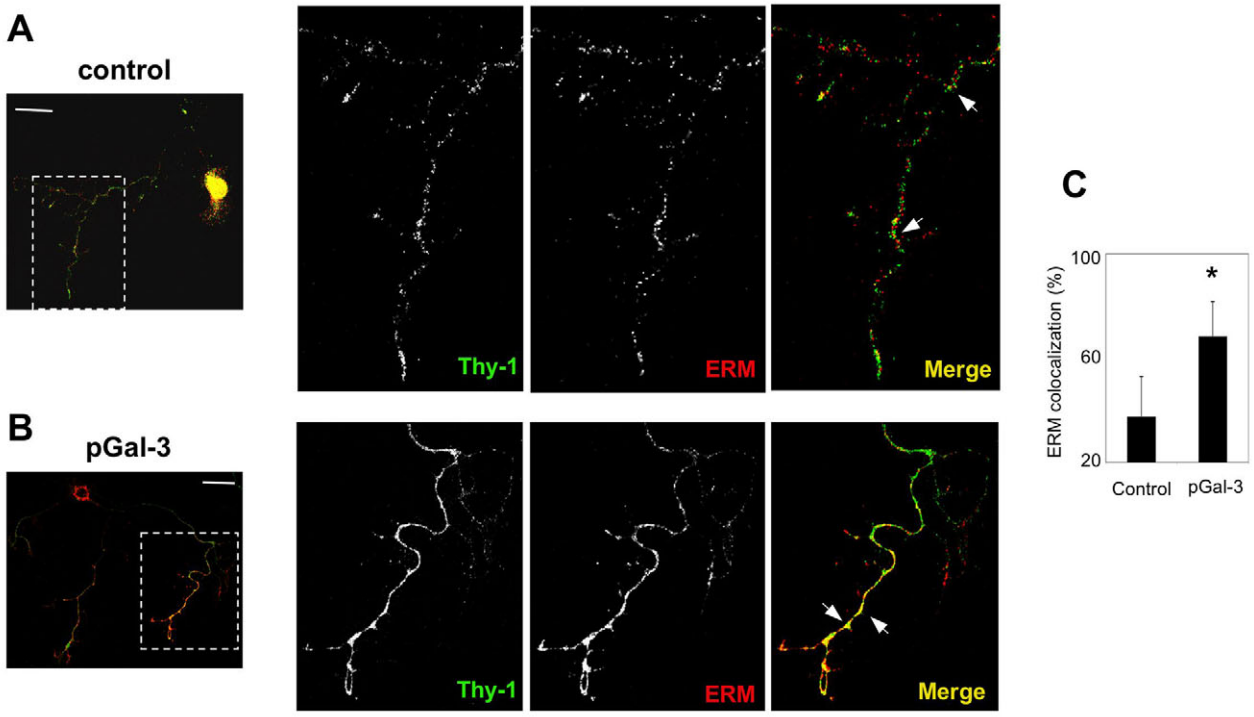

Fig. 7. Bound pGal-3 displaces ERMs to Thy-1-containing membrane rafts. (**A**,**B**) Hippocampal neurons were cultured for 72 hours on PLL (A) or pGal-3-covered coverslips (B). Cells were fixed and immunolabelled with anti-ERM and anti-Thy-1 antibodies. After washing, cultures were incubated with fluorophore-conjugated secondary antibodies and mounted. Arrows indicate colocalizing ERM and Thy-1 clusters. Scale bars: 10 µm. (**C**) The percentage of L1 clusters colocalizing with Thy-1 clusters is significantly higher in neurons cultured on pGal-3 than in controls. Values are mean ± s.d. of three experiments (20 fields per experiment; **P*<0.001).

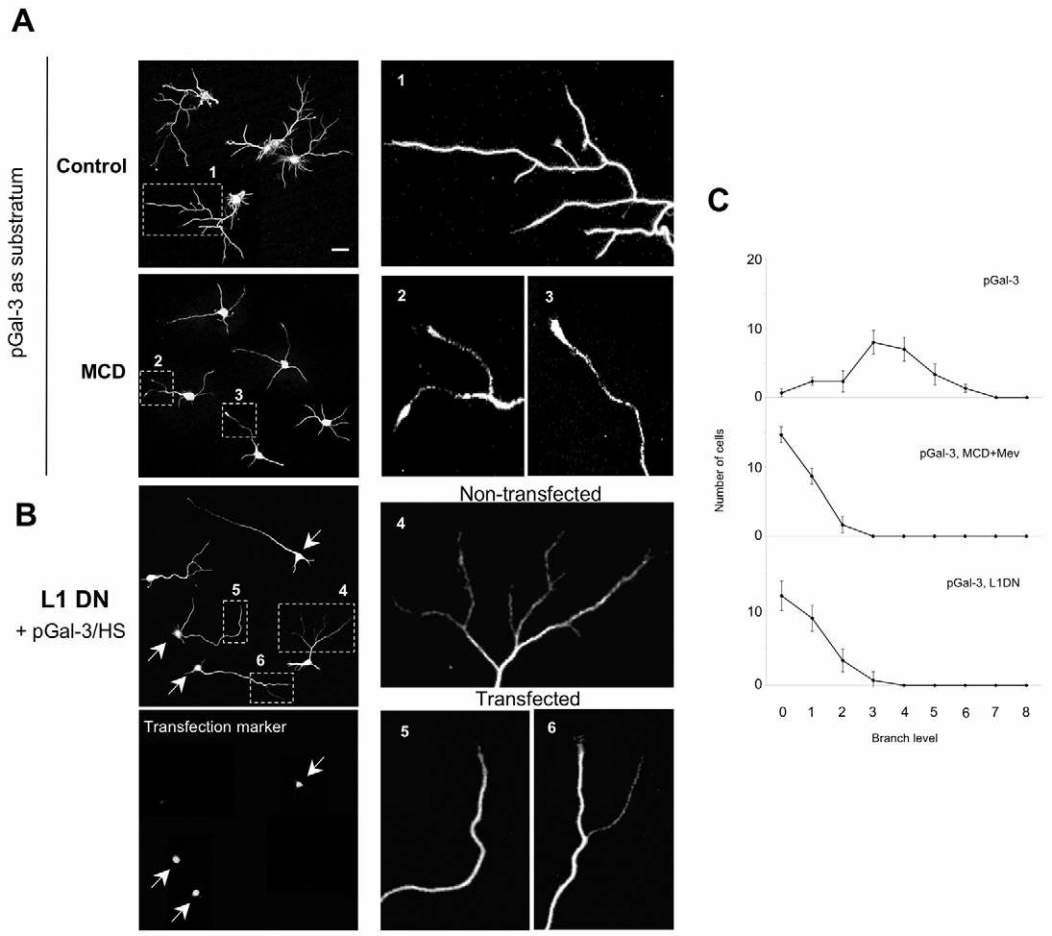

Fig. 8. Dominant-negative L1 and perturbed raft formation impair pGal-3-induced branching. Neurons were fixed and immunolabelled with anti-α-tubulin antibodies. (**A**) MCD was added to 1 DIV neuron cultures and incubated for further 48 hours to lower membrane cholesterol. (**B**) Neuroblasts were nucleofected in suspension with DN-L1 (see Materials and Methods for details), plated and processed after 3 DIV. (**C**) The effect of the two treatments on branching in populations of neurons (see Fig. 1). Results are mean ± s.d. of three experiments (25 neurons per experiment). Scale bars: 10 μm.

pGal-3, L1 is displaced to raft domains, inducing the local recruitment of cytoplasmic ERMs. This pGal-3–HS-induced enrichment of actin-regulating machinery in discrete zones of the plasma membrane is compatible with the formation of branching points at these sites.

Discussion

Neuronal glycoproteins that are involved in differentiation and known for homophilic interactions can also interact with endogenous lectins via glycan chains to initiate signalling. Branch-end epitopes, formed by the letters of the sugar alphabet, thus establish versatile biochemical signals (Rüdiger and Gabius, 2009). It is possible that post-translational modifications of these effectors, here a galectin, may act as a switch for their bioactivity. We herein present the first evidence for modulation of extracellular function of Gal-3 by phosphorylation, using cultures of hippocampal neurons as a model.

Cellular effects triggered by galectins are known to depend on how these lectins are presented to the cell surface. We have previously shown that a chicken prototype galectin was able to accelerate in vitro axon growth when either in solution or bound to the substratum, whereas both these modes of presentation produced the opposite effect on neuritogenesis (Kopitz et al., 2004). By contrast, the chimera-type Gal-3, when supplied with fibronectin to the tumour cell surface, favours carbohydrate-mediated interaction and clustering of integrins, regulating focal adhesion dynamics (Goetz et al., 2008). In this context, we show that branching induction or axon growth induced by Gal-3 occurred only when the protein was adsorbed to the surface or when in complexes with HS (Fig. 1). This suggests that exposure of neurons to Gal-3–HS complexes could resemble the situation in vivo, where the interaction of different forms of Gal-3 with ECM components may stabilize and/or arrange the lectin to engage in protein-carbohydrate and/or protein-protein binding with distinct partners (Gabius, 2006; Gabius, 2008; Smetana et al., 2006).

Several glycoproteins are candidates as counter-receptors on the cell surface. Neuropilin-1, a receptor for the guidance cue semaphorin3A, regulates dendritic branching (Morita et al., 2006), and a glycoform has been identified recently as a galectin-1 ligand (Hsieh et al., 2008). Considering that Gal-1 and Gal-3 display common affinities for β-galactosides including ganglioside GM1 and the *N*-glycans of asialofetuin (Ahmad et al., 2002; André et al., 2005; Dam et al., 2005; Hirabayashi et al., 2002), neuropilin-1 appears to be a suitable candidate to mediate the induction of branching by bound pGal-3. However, no evidence for an interaction

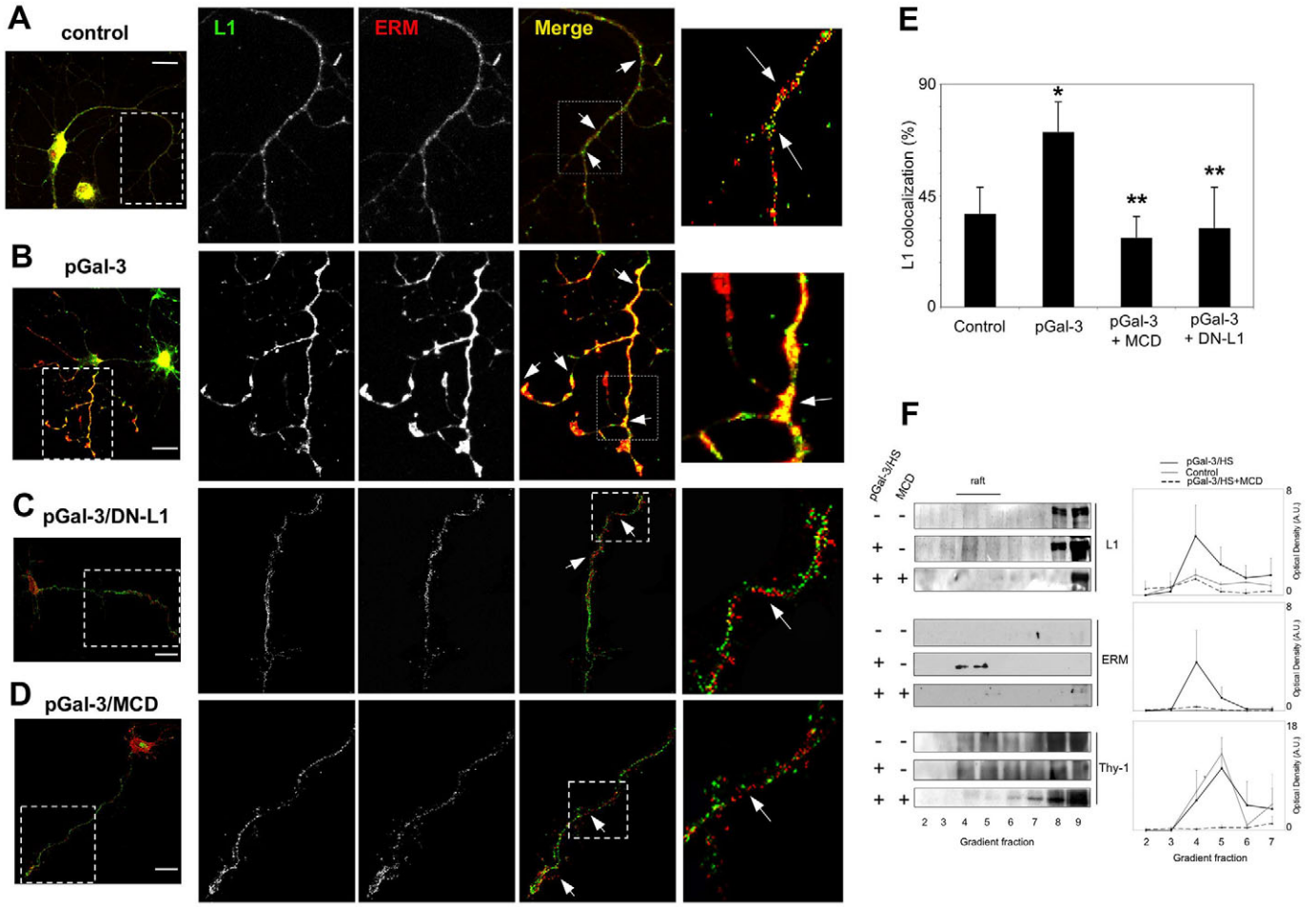

Fig. 9. Bound pGal-3 induces local ERM and L1 colocalization. Hippocampal neurons were cultured for 72 hours on PLL (**A**) or pGal-3-covered coverslips, untransfected (**B**), transfected with DN-L1 plasmid (**C**) or in the presence of MCD for the last 48 hours (**D**). Cells were fixed and immunolabelled with anti-ERM and anti-L1 antibodies. After washing, cultures were incubated with fluorophore-conjugated secondary antibodies and mounted. Arrows indicate colocalizing ERM and L1 clusters at branching points along the axon. (**E**) The percentage of L1 clusters colocalizing with ERM is significantly higher in neurons cultured on pGal-3 than in controls. MCD treatment or DN-L1 expression results in colocalization values similar to those of the control. Values are mean ± s.d. of three experiments (20 fields per experiment; **P*<0.001 vs control; ***P*<0.001 vs pGal-3). (**F**) N1E neuroblastoma cells (treated with MCD where indicated) were cultured for 7 days on Gal-3- or pGal-3-covered dishes. Cell membranes were extracted with a cold solution of 1% Triton X-100 and fractionated in sucrose gradients by ultracentrifugation. Fractions were probed by western blot for L1, ERM and Thy-1. The quantity of protein present in each fraction as measured by densitometry is given in the graphs on the right. In pGal-3–HS-treated cells, L1 and ERM are enriched in fractions 4-5, which corresponds to Thy-1-containing raft fractions (Thy-1 panels). MCD treatment perturbs Thy-1 raft formation and abolishes the pGal-3–HS recruitment effect. Results are mean + s.d. of three experiments. Scale bars: 10 μm.

between pGal-3 and neuropilin was observed (Fig. 5A). Similar results were obtained for β1-integrin (Fig. 5A), although its glycans can also interact with galectins and is involved in dendrite branching and maintenance (Becker et al., 2003; Goetz et al., 2008; Moresco et al., 2005; Poinat et al., 2002).

In contrast to neuropilin-1 and β1-integrin, L1 is sorted to the axons early in neuron differentiation (van den Pol and Kim, 1993), and its clustering with antibodies or by homophilic L1-L1 binding induces a highly branched axon phenotype (Dickson et al., 2002), similar to the one produced by bound pGal-3 (Fig. 4). However, the reactivity of galectin with L1 has, so far, been found to be rather weak (Probstmeier et al., 1995). Since glycosylation is subject to subtle and dynamic regulation, which can influence the strength of molecular contacts, we investigated interactions in this cell system by immunoprecipitation, including a reversible cross-linking step and, indeed, identified significant coimmunoprecipitation of Gal-3 and L1 (Fig. 5). Importantly, pGal-3 shows stronger interaction with L1 than Gal-3, and this was corroborated by inverse immunoprecipitation with anti-L1 antibodies (Fig. 5B). Proteolytic truncation will irreversibly switch off this activity, a new functional dimension of this physiological activity by matrix metalloproteinase-2 to -9, seen for example in breast cancer and terminal differentiation of chondrocytes (Lohr et al., 2008; Nangia-Makker et al., 2007; Ortega et al., 2005).

Axon growth mediated by L1 interaction depends on L1 association with DRMs within the growth cones. Local perturbation at the growth cone, targeting the ganglioside GM1 by micro-scale chromophore-assisted laser inactivation (micro-CALI), stops axon elongation and the growth cones undergo a drastic change of morphology, from lamellipodia to filopodia (Nakai and Kamiguchi,

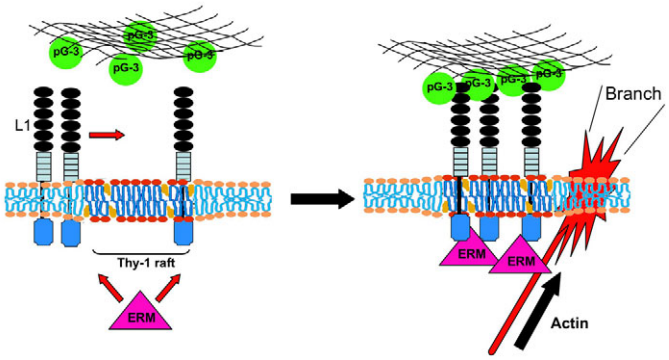

Fig. 10. Proposed model for induction of local branching by pGal-3 presentation. Components of the extracellular matrix would present pGal-3 to the axon membrane in an optimal spatial disposition to interact with L1, resulting in its displacement to Thy-1-containing rafts. The phosphorylated N-terminal section of the lectin is essential, suggesting a potential for concomitant binding via the carbohydrate-recognition domain and the phosphorylated N-terminal section. The local accumulation of L1 by pGal-3 would trigger the recruitment of membrane-actin linkers (ERMs), creating a zone of highly dynamic actin cytoskeleton, and facilitating the onset of a new branch.

2002). Interestingly, the immunoreactivity of Thy-1, a GPI-anchored protein associated with DRMs (Ledesma et al., 1998), is not affected by the described micro-CALI targeting ganglioside GM1 (Nakai and Kamiguchi, 2002). Growth cone perturbation induces a modification of the lipid to protein balance of the membrane domains that impairs L1-mediated growth signal transduction and favours filopodia formation. Consistent with this mechanism, our results indicate that interaction with pGal-3–HS brings L1 into extensive association with Thy-1-containing axon membrane domains (Fig. 6). In support of this conclusion, impaired L1 signalling by expression of the dominant-negative form of L1, or perturbed raft formation by lowering membrane cholesterol, eliminates the effects of pGal-3 in complexes with HS. In consequence, local L1 enrichment would create a platform where ERMs are recruited (Figs 7 and 9) to favour a branching point, and to activate the formation of a new protrusion (Fig. 10).

In vivo, exploratory filopodia formation and axon branching are associated with nerve development and, importantly, with nerve-regenerative activity after injury. In the nervous system Gal-3 is expressed, among others, by activated Schwann cells after peripheral nerve injury (Reichert et al., 1994) and by olfactory ensheathing cells, as we (data not shown), and others (Storan et al., 2004) have observed. Taking into consideration that both the peripheral nervous system and the olfactory system (CNS) show nerve regeneration capacity, it is tempting to speculate that induction of Gal-3 expression in axon-ensheathing cells could correlate with regenerative activity in the nervous system. In support of this idea, neither injury-activated astrocytes nor oligodendrocytes express Gal-3 (Kuklinski et al., 2000), coincident with the lack of regeneration in the CNS. Whether Gal-3 phosphorylation could act as a signal for a role in nerve regeneration, by facilitating axon branching, is the object of ongoing research.

In conclusion, the described strategic combination of post-translational modification and presentation by ECM molecules probably accounts for the specific activity level of Gal-3 in axon regulation. In particular, the presentation as pGal-3–HS complex induces the recruitment of L1 and ERM actin-regulating machinery to discrete zones of the plasma membrane, where emergence of new branches is prompted. We propose that the spatial distribution of pGal-3 in the extracellular matrix, at least in part, can determine the extent of axon branching and the position where branches will protrude, processes decisively involved in nervous system development and, of clinical relevance, in regeneration.

Materials and Methods

Preparation of the Gal-3 forms and complexes with heparan sulphate

Human Gal-3 was obtained by recombinant production, purified by affinity chromatography on lactosylated Sepharose 4B, and controlled for purity by one- and two-dimensional gel electrophoresis and mass spectrometry. Proteolytic truncation by collagenase, phosphorylation by casein kinase 1, and biotinylation were carried out with quality controls and activity assays by solid-phase and cell binding as described previously (André et al., 2006; Kübler et al., 2008) (supplementary material Fig. S1). Complexes of heparan sulphate proteoglycans (HS isolated from basement membrane of EHS mouse sarcoma; Sigma-Aldrich, St Louis, MO) and Gal-3 forms were prepared by incubating HS and the galectin (1:5; w/w) in PBS, for 1 hour, at 37°C. Solutions of the complexes were added to the neuronal cultures at a final concentration of 25 μg galectin/ml.

Antibodies

For immunocytochemistry and western blotting, the following antibodies were used: mouse monoclonals anti-α-tubulin (Sigma-Aldrich), anti-Thy1 (Serotec), and anti-β1-integrin (BD Biosciences); rabbit polyclonals anti-ERM (Cell Signaling) and anti-galectin-3 (André et al., 2004); goat polyclonals anti-L1 and anti-neuropilin (Santa Cruz Biotechnology). F-actin was detected with Alexa-Fluor-568-conjugated phalloidin (Molecular Probes). Secondary antibodies for immunocytochemistry were Alexa-Fluor conjugated, and HRP conjugated for western blotting (all from Molecular Probes).

Cell culture, immunofluorescence and morphological analysis

Rat embryo hippocampal neurons were cultured as described previously (Goslin et al., 1998). Cell suspensions were plated onto poly-L-lysine (PLL) or PLL- and galectin-covered glass coverslips in minimal essential medium (MEM) supplemented with 10% horse serum (MEM-HS). PLL-galectin-covered coverslips were prepared by incubation of PLL-covered coverslips with recombinant galectins (1 mg/ml) for 12 hours at room temperature. After cell attachment (6-8 hours), coverslips were transferred to Petri dishes containing MEM supplemented with N2 and galectins in solution at 25 μg/ml (final concentration) were added. For biochemical purposes, cortical neurons from E18 rat embryos or N1E neuroblastoma cells were cultured on PLL- or galectin-covered plastic Petri dishes containing MEM-HS medium.

For morphological analysis cells were fixed and immunolabelled with mouse monoclonal anti-α-tubulin and fluorophore-conjugated anti-mouse secondary antibodies. Random images (10 fields per coverslip) were taken from each experiment using an epifluorescence microscope (Leica DM5000B), and cell parameters were measured up to a total of 50 cells per experiment (three experiments). Only cells in clusters or those with inter-crossed axons were excluded from analysis because of the difficulty of unequivocally tracking the axon trajectory. Axons were measured using ImageJ software (NIH). Results were subjected to Student's *t*-test-based statistical analysis.

Axon branches were counted manually, in the same images. Branch level is defined as follows: axon only =level 0; branch from axon =level 1; branch from level 1 =level 2; etc. Control experiments were performed in the presence of 25 mM lactose (LAC). Results are means ± s.d. of three experiments (25 neurons per experiment).

Parallel-substrate-challenge assays for axonal guidance were set up by coating the glass coverslip surface with the various Gal-3 forms in parallel stripes. Silicon matrices with parallel micro-channels were used as described previously (Baier and Klostermann, 1994). Briefly, solutions containing Gal-3 forms (1 mg/ml) and FITC (1 μg/ml; Sigma-Aldrich) were injected into the matrices and incubated for 3 hours at room temperature. Striped substrata covered with laminin (1 mg/ml; Sigma-Aldrich) and FITC, or FITC alone, were used as positive and negative controls, respectively.

Antibody-induced patching and complex binding

For antibody-induced patching, cultured neurons were first washed with PBS and then incubated in CO_2-free MEM medium for 45 minutes at 12°C with anti-Thy-1 and anti-L1 primary antibodies. After another brief washing, the cells were incubated under the same conditions with fluorophore-coupled anti-species antibodies to favour molecule patching. Coverslips were then fixed with 4% paraformaldehyde [with the exception of ERM labelling, for which 10% trichloroacetic acid at 4°C was used (Hayashi et al., 1999)], and mounted.

Photomicrographs were taken on a confocal microscope (Leica SP5) and analyzed with ImageJ software. For cluster sizing and colocalization measurements in antibody-induced patching experiments, ten fields from ten different images were analyzed. Briefly, Thy-1 and L1 channels were separated and thresholded to define positive signal clusters. Colocalizing areas were calculated by superimposing positive cluster maps (Abad-Rodriguez et al., 2004).

To check the binding to axons of Gal-3 forms in complex with HS, hippocampal neurons cultured for 72 hours were incubated for 1 hour at 12°C, in buffered MEM medium, with biotinylated Gal-3 forms (25 μg/ml) in complexes with HS. Cells were then washed and further incubated under the same conditions with FITC-conjugated streptavidin. Cultures were then fixed, mounted and imaged with a confocal microscope.

L1 dominant-negative constructs, transfection and membrane cholesterol extraction

cDNA of the dominant-negative form of L1 (L1-1151Y>A) (Cheng et al., 2005) cloned in a pcDNA3 vector was purchased from Addgene (plasmid 13269). Neurons in suspension were transfected using the Nucleofector system (Amaxa, Gaithersburg, MD) according to the manufacturer's instructions. Cherry nuclear was used as transfection reporter.

For low-membrane-cholesterol experiments, MCD was added to 1-day in vitro (DIV) neurons or N1E cultures to a final concentration of 0.5 mM or 500 mM, respectively. Cells were processed at 3 DIV for immunocytochemistry or at 7 DIV for membrane fractionation.

Membrane extracts preparation and fractionation

Neuron cultures were scraped into cold MBS buffer (25 mM MES, 150 mM NaCl, pH 6.5) supplemented with protease inhibitors (pepstatin, leupeptin, antipain, chymostatin, each at a final concentration of 25 μg/ml). Suspended cells were lysed by ultrasonication, homogenized by ten passages through a 22-gauge syringe, and the resulting extract was centrifuged for 10 minutes at 900 ***g***. Supernatants were considered as total extracts. A further centrifugation was performed at 10^5 ***g*** for 1 hour at 4°C to pellet the membrane fraction. Protein concentration was quantified by the BCA method (Bio-Rad). Membrane pellets were then extracted in MBS buffer containing CLAP and 1% Triton X-100. After 60 minutes of incubation at 4°C, the suspensions were added to 60% sucrose in MBS and a sucrose step gradient was overlaid (35% and 5% sucrose). After centrifugation at 155,000 ***g*** (SW-40 rotor, Beckman) for 18 hours at 4°C, fractions were collected from the top of each tube and analyzed by western blotting.

Cross-linking and immunoprecipitation

Cortical neurons cultured in plastic dishes at high density were incubated for 2 hours, at 12°C, in Locke's solution, with Gal-3 or pGal-3 (25 μg/ml) in complex with HS. Cultures were then incubated with the cross-linking reagent DTSSP (Pierce) following the manufacturer's instructions, quenched with Tris buffer (pH 7.5) and scraped into cold lysis buffer (PBS, 5 mM EDTA, 0.5% Triton X-100). After centrifugation, supernatants were incubated with anti-Gal-3 or anti-L1 antibodies at 4°C for 18 hours, and precipitated with protein-A- or protein-G-Sepharose. The immunoprecipitated complexes were separated by SDS-PAGE and subjected to western blotting for β_1-integrin, neuropilin-1 and L1.

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http://jcs.biologists.org/cgi/content/full/123/5/671/DC1

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