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Research report

# Hippocampal neurons and recombinant galectins as tools for systematic carbohydrate structure–function studies in neuronal differentiation

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**Abstract**

Membrane glycoconjugates play a central role in neuronal interactions and regulation. To define the precise links between membrane polysaccharides and neuronal functions, two main requirements must be fulfilled: (1) the availability of molecular tools able to finely discriminate among carbohydrate structures and (2) the use of an experimental system suitable for systematic and quantitative studies of particular neuronal processes. In this work, we used two chicken proto-type galectins, i.e., monomeric CG-14 and dimeric CG-16, with very similar carbohydrate affinities, and rat hippocampal neurons in culture to quantitatively measure the involvement of carbohydrate–protein interaction in axonal growth and directionality, neurite sprouting and axon regenerative capacity after section. CG-16 potently stimulated axonal growth and guidance. Neurite sprouting was enhanced by immobilized CG-16 and, notably, reduced by lectin in solution. Overall, cross-linking CG-16 invariably excelled CG-14 in these functional assays, although none of them were able to improve axon regenerative capacity when compared to mammalian galectin-1. Our results demonstrate the potential of the experimental set-up to perform a systematic study of galectin functionality in neuronal differentiation. In view of the concept of the sugar code, the presented results indicate that biological effects triggered by glycan binding engaging an endogenous lectin can be modulated by carbohydrate affinity and/or by other factors like differential cross-linking capacity.


*Theme:* Development and regeneration
*Topic:* Process outgrowth, growth cones, and sprouting

*Keywords:* Axonal growth; Axonal regeneration; Neuritogenesis; Galectin; Protein–carbohydrate interaction, sugar code

## 1. Introduction

Spatial accessibility and structural diversity are essential prerequisites for cell surface epitopes to play a role in cellular interactions. These conditions are fulfilled for β-galactosides and their multiple derivatives at branch ends of glycoconjugates, allowing them to act as sensors for sugar receptors (lectins) [13,14]. Galectins are a major family of lectins homing in on distinct β-galactoside structures. They are defined by their common carbohydrate specificity to a β-galactoside core and the jelly-roll-like folding [13,26]. Currently, 14 family members are known in mammals, and monitoring by RT-PCR analysis or immunohistochemistry has revealed a complex expression pattern for diverse cell types [7,25,28]. In the nervous system, only mammalian proto-type galectin-1 has been thoroughly studied. Currently available data regarding this lectin establish a clear link between its carbohydrate-binding capacity and its physiological function in the nervous system [40]. In fact, galectin-1 is selectively expressed in sensory neurons and motoneurons [20] and it

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mediates the contacts to laminin/merosin and to olfactory neurons [8]. It has been shown as well that galectin-1 stimulates neurite outgrowth in an olfactory neuron line [27] and regulates sorting and fasciculation of olfactory axons [22,32,37] The capacity of galectin-1 to regulate cell growth was further substantiated with SK-N-MC neuroblastoma cells. Notably, this activity could be blocked by chimera-type galectin-3 on the level of ligand binding, an instructive case study for functional divergence [23]. In this case, the pentasaccharide chain of ganglioside GM1 served as common galectin target, in fact for mammalian and for avian galectins, a notable result for the course of our study [1,36]. The work on galectins from different mammals reveals only minor sugar specificity differences. It is one open question whether and to what extent prototype galectins (one carbohydrate recognition domain per protomer in mono- or dimers) share functionality. According to these data, it is a promising concept that galectins act as effectors of neuronal functions and that, due to their carbohydrate-binding specificity, they can be used as optimal tools for the systematic dissection of β-galactoside involvement in neuronal development. To address this issue, the selection of a suitable model system serves two means: to compare functional profiles for structurally well-defined lectins and to validate the whole experimental system for further studies. Toward these ends, we selected cultures of rat hippocampal neurons and the two proto-type chicken galectins (CG) for the quantitative analysis of neuritogenesis, axonal growth and axonal regeneration.

The two chicken galectins, CG-14 and CG-16, designated according to their molecular weights, arose by gene duplication of an ancestral gene at a time close to the evolutionary divergence of birds and mammals [34]. The diverging sequences still maintain 48% identity [34]. Of note, this degree of similarity is also reached in comparison to mammalian galectin-1 and between different mammalian galectins and it translates into similar topologies of the binding site (André S. et al., in preparation). CG-14 and -16 harbor close similarity in ligand selection, as measured by chemical mapping [36]. For CG-16, comparison of thermodynamics of binding and X-ray structure underscores the similarity seen on the sequence level [4,38,39]. Additionally, CG-14 (monomeric) and -16 (dimeric) represent the two possibilities of quaternary structure for proto-type galectins [3]. Because galectins are secreted and can exert their extracellular functions either from solution or from the extracellular matrix [12,19], we aimed to elucidate the impact of this factor by running our assays with galectins as substratum or added as medium component. Here, we present first evidence for differential effects on neuronal development by membrane carbohydrate interaction with close-related proto-type galectins, and validate the use of hippocampal neurons in culture for the quantitative evaluation of protein–carbohydrate interaction effects on neuronal function.

## 2. Materials and methods

### 2.1. Preparation of the galectins

The presence of both galectins was ascertained in embryonic kidney [38], and the cDNAs were cloned from total kidney mRNA either with suitable primer sets by RT-PCR for CG-14 [29] or by consecutive primer-directed RT-PCR and then 3′-RACE-PCR to complete the terminal sequence section for CG-16. Recombinantly produced galectins were purified as described [11]. Elution buffer included iodoacetamide to stabilize the carbohydrate-binding activity [31]. Labeling and activity assays were carried out as described [1].

### 2.2. Cell culture, immunofluorescence and morphological analysis

Rat embryo hippocampal neurons were cultured as described [15]. They were plated onto poly-L-lysine (PLL) or PLL/galectin-covered glass coverslips in minimal essential medium (MEM) containing N2 supplements. PLL/galectin-covered coverslips were prepared by incubation of PLL-covered coverslips with recombinant chicken galectins (10, 100, 1000 and 5000 μg/ml) for 12 h at room temperature. Galectins in solution were added 6 h after cell plating at 5, 10, 25 and 50 μg/ml (final concentration) and further incubated for 30 h. To test that the galectin binding to neuronal surface was carbohydrate-dependent, 24 and 48 h in vitro hippocampal neurons were incubated with 25 μg/ml biotinyl-CG-14 or biotinyl-CG-16, at 12 °C for 30 min, in the presence or not of various concentrations of lactose. Cells were then washed, incubated with Texas red-conjugated streptavidin (Vector) under the same conditions, fixed and mounted. After the different treatments cells were fixed and immunolabeled with mouse monoclonal anti-tubulin (Amersham). Alexa-conjugated anti-mouse antibodies (Molecular Probes) were used as secondary antibodies. Cells were photographed with a fluorescence microscope (DMIRE2, Leica). Random pictures (10 fields/coverslip) were taken from each experiment and all the cells were considered up to a total of 100 cells/experiment (three experiments). Only cells in clusters were discarded due to the difficulty to unequivocally localize the origin the neurites. Neurite number and length measurements were performed using NIH image software. Obtained values were subjected to Student's *t* test-based statistical analysis.

### 2.3. Parallel-substrate challenge assay for axonal guidance

Galectin coating in parallel stripes was performed on PLL-pretreated coverslips using silicon matrices with parallel micro-channels as described previously [2]. Briefly, galectin solutions (1 mg/ml) and FITC (1 μg/ml) were injected into the matrices and incubated for 3 h at room

temperature. As control, we used a solution of FITC at the same concentration.

### 2.4. In vitro axon regeneration assay

Neurons growing for 48 h in vitro on Cellocate® coverslips (Eppendorf) were localized on grids using a 40× long-distance working lens. The longest neurite was then sectioned by pulling it over the glass surface and orthogonally across, a microinjection glass needle controlled by a micromanipulator (Eppendorf). Distances between cell bodies and section sites were always similar to the second longest neurite of each cell. Neurons (30 cells/experiment, three independent experiments) were photographed before and immediately after transection. Coverslips were then treated with recombinant chicken galectins (25 μg/ml). After 24 h, cells were localized and newly photographed.

## 3. Results

### 3.1. Effects on axonal growth and neuritogenesis

To test whether the recombinant lectins used in this work were binding to neuronal plasma membrane in a carbohydrate-dependent manner, we studied the binding of biotinylated CG-14 and CG-16 [35] in the absence and in the presence of lactose. Both galectins bound to the plasma membrane of living cultured neurons, either at early developmental stages (Fig. 1A, 24 h) or after morphological polarization, staining appearing slightly enriched in the growing axon (Fig. 1A, 48 h). Binding resulted extensively reduced in the presence of 200 mM lactose (Fig. 1A, lactose). These results ascertain the dependence on β-galactoside-specific interactions for CG-14 and CG-16 neuronal surface binding.

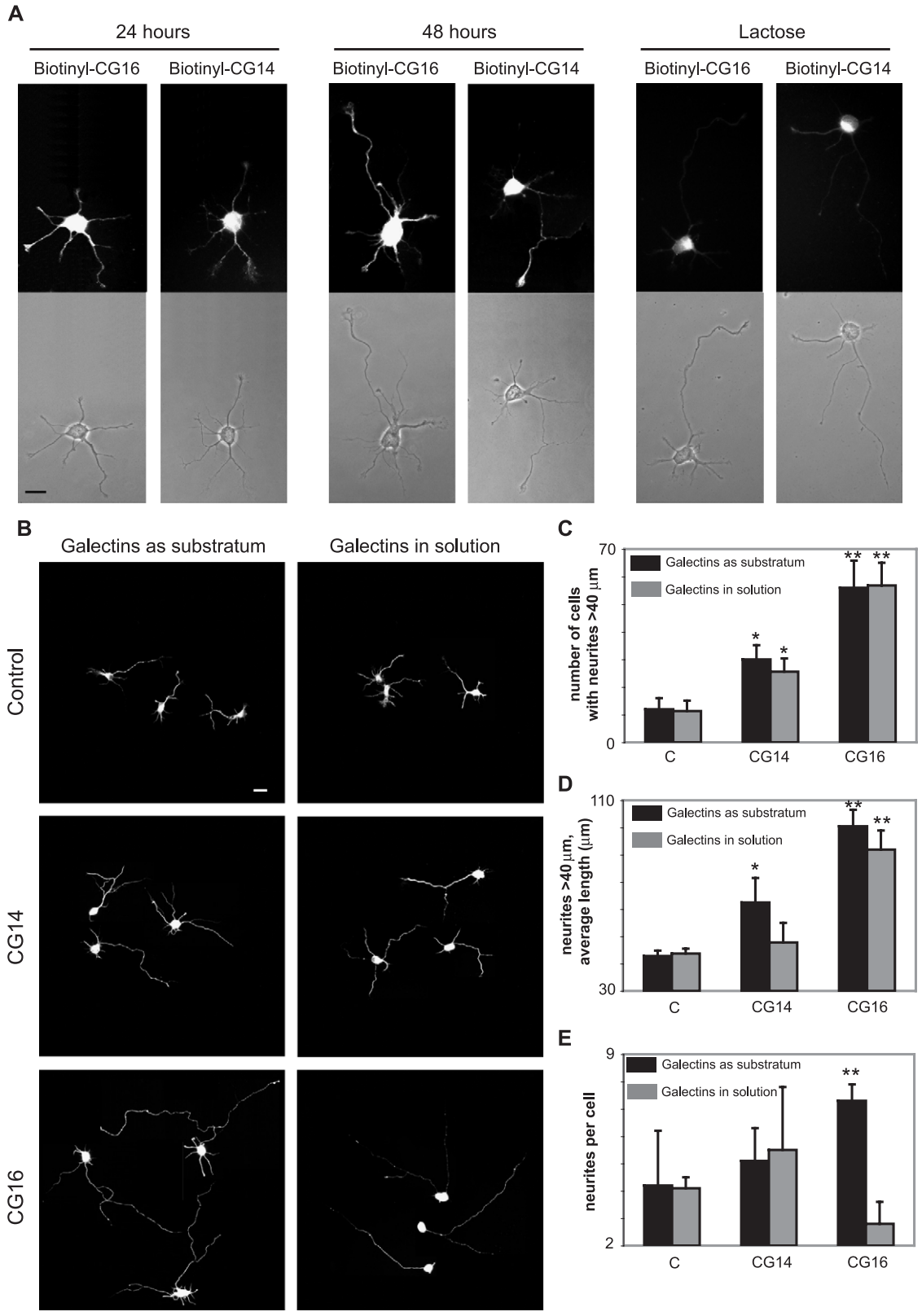
To assess galectin effects on axonal growth and neuritogenesis, we assayed them as substratum or in solution (see Materials and Methods for details). Our assay set-up thus mimics the two modes of physiological galectin presentation, either bound to ECM/cell surface or free in solution after secretion. To check for possible differences in galectin binding to PLL-coverslips, we evaluated the covering by fluorescence microscopy and by a colorimetric assay, using biotinylated recombinant galectins. 10 and 100 μg/ml did not saturate the surface of the coverslips, while 5000 μg/ml produced an irregular covering probably due to the formation of galectin aggregates (data not shown). 1000 μg/ml produced a comparable, homogeneous and saturated covering for both, CG14 and CG16, and was selected as the optimal concentration for the purposes of our study. We detected differential cell responses to galectin presence (Fig. 1B). The monomeric CG-14 as substratum moderately enhanced axonal growth determined by computing the number of neurons with one neurite longer than 40 μm (30±5.2 neurons compared to 12±4 of controls; $p<0.001$. Fig. 1C) and the average length of this group of neurites (62.5±6 μm compared to 42.8±2 μm for the controls. Fig. 1D). The effect appeared somewhat weaker for CG-14 in solution. In fact, although this galectin enhances the number of neurons with long neurites (25.6±4.8; $p<0.001$) they are not significantly longer than in controls (47.8±7 μm). When comparing these data to that of dimeric CG-16, two major differences became evident: CG-16 is a clearly more potent effector than CG-14, and its activity is less dependent on the mode of presentation. On average, 56±9.8 (average length: 90.5±11 μm) and 56.8±8.2 (average length: 81.9±5.9 μm) neurons presented neurites over 40 μm when CG-16 was presented as substratum or in solution, respectively (Fig. 1C,D). The same pattern with one interesting exception emerged for the parameter of neurite sprouting. Monomeric CG-14 did not enhance significantly the number of neurites per cell regardless of the mode of presentation (Fig. 1E). On the other hand, surface-presented CG-16 was very active in this respect. The presence of the dimeric lectin in solution, however, seems to maintain neuritogenesis below the control level, although these differences are in the limit of statistical significance ($p<0.05$). Interestingly, similar qualitative results were obtained with recombinant galectins in solution at 10 μg/ml while no effect was detected at 5 μg/ml (data not shown). An amount of 50 μg/ml or higher produced aggregates and neuronal toxicity. Having hereby documented that the two model galectins exert non-identical roles on axonal growth and that CG-16 as substratum was very active to stimulate neuritogenesis, we next addressed the issue as to whether these galectins might play any role in the directionality of the axon growth.

### 3.2. Effects on guidance of axon growth

The coverslips in these assays were processed to establish a regular pattern of parallel lines with poly-L-lysine and galectin as substratum. FITC was used to label the stripes containing the galectin (Fig. 2A, clear stripes). Each axon thus had the choice to cross a line or to grow along one of the lines, resulting in three possibilities. In controls with stripes covered by PLL and FITC only, axons disclosed no notable preference: 14±1 and 10±2 axons followed a line of PLL or FITC, respectively, and about 16±1.6 crossed a line (Fig. 2B, FITC). CG-14 caused a slight but significant shift of this parameter with an increase of the number of axons growing on the galectin substratum (15.2±1.4; $p<0.01$) and a decrease of their growth on PLL (7.6±1.2, $p<0.01$), indicating a preference for the tested molecule. The extent of the lectin-dependent effect was clearly higher for CG-16 (Fig. 2B). The majority of the axons tended to grow along the stripes covered with it (26.4±1.6; $p<0.001$) rather than on PLL (5.6±0.5; $p<0.001$) or across the lines (8±0.8; $p<0.001$). Considering that CG-16 markedly enhances outgrowth of axons and is a favorable substratum to guide them, we next wondered whether it might be able to modify their regenerative capacity after transection.

### 3.3. Effects in axonal regeneration

Axotomy was routinely performed leaving the proximal stump with a length similar to that of the second longest extension. The rationale for doing this is to return the neuron to a stage in which all processes would have similar chances to become the new axon [15,33]. As typical hippocampal neurons in culture present four to five



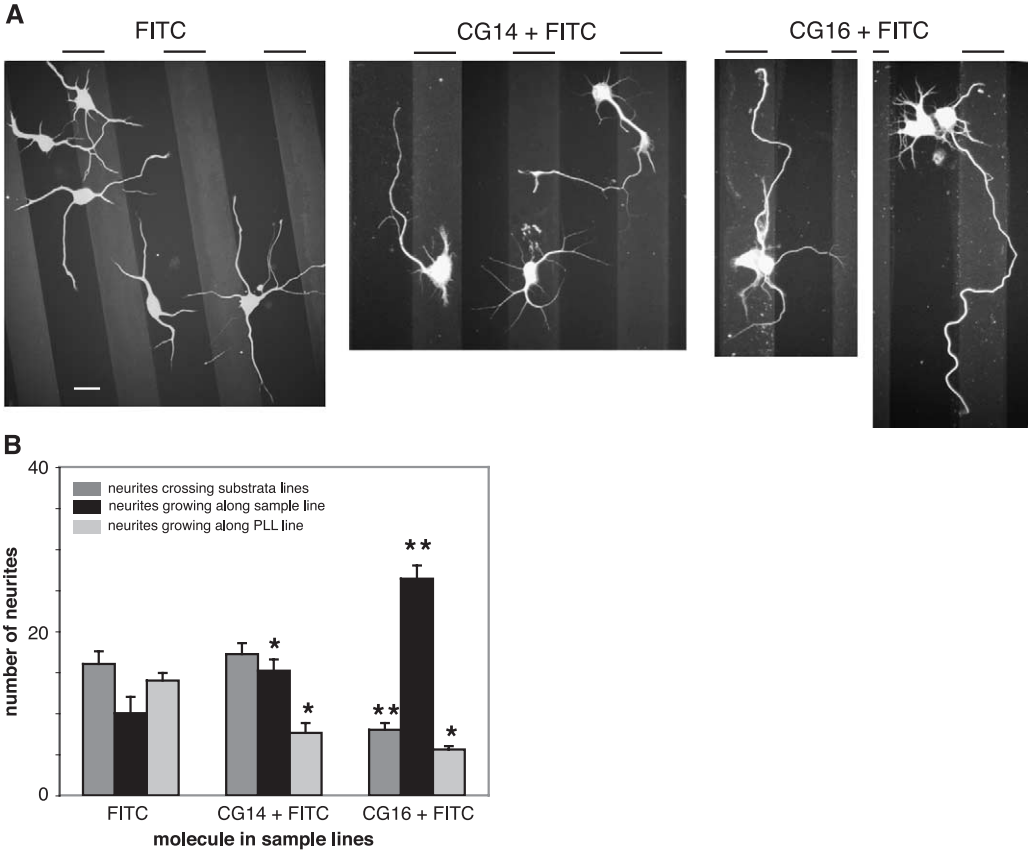



Fig. 2. Axon guidance capacity of chicken galectins. (A) Hippocampal neurons cultured for 48 h on coverslips presenting parallel stripes of FITC together with the indicated galectin or FITC alone as control (light stripes). Anti-tubulin staining was performed to visualize the cells. Neurons extend long axons along CG-16 stripes (right) while they do not show any preference for CG-14 (central) or FITC alone (left). (B) Number of axons growing along either substratum or across both of them. The majority of the axons grow along CG-16 substratum while CG-14 effect is very weak. Data are mean+S.D. values of three experiments (40 cells/experiment); *$p$<0.001 in comparison with control; **$p$<0.001 in comparison with control and CG-14. Scale bar, 10 μm.

neurites (please see also Fig. 1E), the random nature of the decision explains why about 22% (an average of 6.6±1.1 axons out of 30) of the lesioned axons regenerated in control neurons (Fig. 3A, upper panels, and B). CG-14 did not change this percentage (6.7±0.9 axons), and CG-16 in this experimental setting was only slightly, although not significantly, active (8.6±0.6 axons; $p$<0.05; Fig. 3A, central panels, and B). Because oxidized human galectin-1 had been reported to enhance axonal regeneration from transected nerve sites of adult rat dorsal root ganglion explants [21], we tested human galectin-1 as lectin in parallel assays. These experiments were also prompted by the fact that galectin-1 is a major cellular receptor for $GM_1$, a ganglioside that engenders regrowth of lesioned axons [24,33,36]. Human galectin-1 (25 μg/ml), indeed, significantly skewed the balance in favor of regrowth as 12±1.9 of the lesioned axons regenerated ($p$<0.01; Fig. 3A, lower panels, and B).

## 4. Discussion

Rat hippocampal neurons develop in vitro following a well-defined temporal sequence of events, from initial neurite formation after plating (stage 1) to the fully polarized phenotype (stage 5) [10]. This advocates their use to quantitatively analyse different aspects of neuronal development. Indeed, we establish this culture as an experimental system suitable to assign neuronal functions to particular galectins and their respective interactions with

Fig. 1. Effect of chicken galectins on axonal outgrowth and neurite generation. (A) Hippocampal neurons cultured for 24 and 48 h labeled with biotinyl-CG-14 and biotinyl-CG-16. Both galectins bound to the neuronal membrane showing a slight enrichment at distal part of the axons after 48 h. Lactose inhibition (200 mM, see Materials and methods) ascertained carbohydrate-dependent binding. (B) Hippocampal neurons cultured for 36 h on galectin-coated coverslips (left) or treated with galectin solutions (right) and labeled with anti-tubulin antibodies. (C) Number of cells showing a neurite longer than 40 μm depending on the presence of chicken galectins. (D) Average length of neurites longer than 40 μm. Note that CG-16 promotes dramatically axonal growth while CG-14 only produces a weak effect. (E) Average number of neurites per cell. CG-16 acting as substratum significantly enhances neuritogenesis. In C, D and E, a total of 300 cells were evaluated in three independent experiments (100 cells/experiment). Data are mean+S.D. values of the three experiments; *$p$<0.001 in comparison with control; **$p$<0.001 in comparison with control and CG-14. Scale bar, 10 μm.

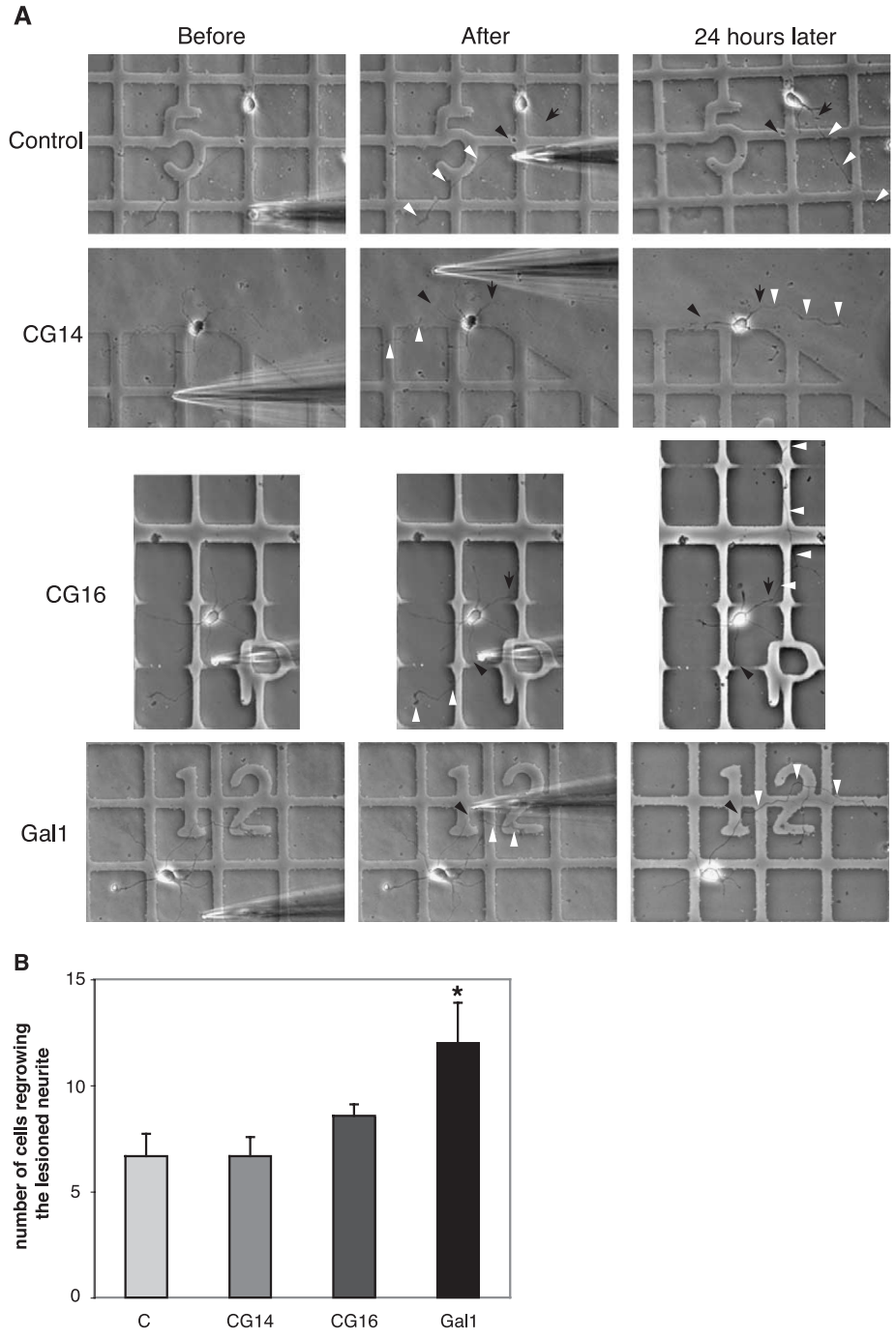



Fig. 3. Axon regeneration is not induced by chicken galectins. (A) Hippocampal neurons plated on Cellocate® coverslips were grown for 48 h. The axon was then sectioned with a glass needle (see Materials and methods for details) and treated either with chicken galectins or with Gal-1 for comparison. Neurons were photographed before, immediately after and 24 h after transection. Black arrowheads point to the site of the lesion, black arrows indicate the neurite growing after 24 h; white arrowheads highlight sectioned and growing neurite. (B) Number of neurons which axon regenerated after lesion. A total of 90 cells were evaluated in three independent experiments (30 cells/experiment). Data are mean+S.D. values $*p<0.001$ in comparison with control, CG-14 and CG-16.

specific carbohydrate structures. In fact, our results support the concept that protein–carbohydrate interactions play essential roles in neuronal developmental processes, while they highlight the fact that binding of β-galactosides to galectins is per se not sufficient to elicit a biological effect.

For instance, axon growth and guidance are facilitated by dimeric CG-16 while the monomeric homologue, CG-14, showed significantly weaker activity. Considering that there are no significant differences in galectin binding to the PLL-coverslips, this result suggests that not only carbohydrate binding but also carbohydrate cross-linking capacity can modulate the effects exerted by the lectin–glycan interactions. Admittedly, we cannot rule out the possibility that slight differences between CG-16 and CG-14 sugar-binding

specificities are responsible for the differential effects. In fact, based on chemical mapping with ligand derivatives and a ligand panel of free oligosaccharides, quantitative discrepancies in affinity toward certain determinants were detectable [17]. In this case, considering the rather similar staining pattern and its blocking by lactose, cell surface target sites appeared to be comparable. In addition, the high level of sequence identity supports close relationship between CG-16 and CG-14. It will be of interest to assess the impact of the cross-linking capacity of these galectins, for example, by mutational engineering to turn a monomer into a dimer and vice versa.

We also observe that CG-16 stimulates neuritogenesis when presented as a substratum. In contrast, when presented in solution neuritogenesis is slightly impaired. For comparison, monomeric CG-14 does not influence neurite sprouting. Neuritogenesis is dependent on dynamic changes in the local actin cytoskeleton, triggered through the RhoA/ROCK/profilin IIa small GTPase pathway [9]. Galectin-1 regulates this pathway in glioblastoma cells resulting in modifications of the actin network [6]. Fittingly, preliminary results from our group indicate that galectin-1 as substratum led to a strong increase of neurite number rendering a connection between glycan–substratum interaction and actin rearrangement likely to be pursued in future studies.

A factor which often leads to misinterpretations of galectin function is the reduction of their sugar binding by oxidative events [18,30]. To stabilize the carbohydrate-binding activity of the galectins used in this work, we treated them with iodoacetamide during affinity elution and the maintenance of their activity was routinely ascertained by haemagglutination and solid-phase assays. It has been shown that oxidized galectin-1, with no carbohydrate-binding capacity, is able to promote peripheral nerve regeneration [21]. In our study, galectin-1, acting as a lectin, promotes axonal regeneration of hippocampal axons in vitro. The fact that hippocampal neurons are components of the central nervous system may be basic to explain this different behavior of galectin-1, considering the general physiological differences between peripheral and central nervous systems and, in particular, their distinct capacities for regeneration. At any rate, the use of iodoacetamide-stabilized galectins and the reduction of their binding to neurons in presence of lactose indicate that our results can be attributed to galectin–carbohydrate interactions.

Interestingly, CG-16 does not enhance axon regeneration as galectin-1 does. Although CG-16 and this mammalian homologue are both cross-linking modules [5,16], it is likely that differences in carbohydrate affinity could account for the observed opposite effect. In fact, the high density of $GM_1$, one of the main galectin-1 ligands at the neuronal cell surface, enhances the regenerative capacity of hippocampal axons [33]. Moreover, CG-16 binds inefficiently to $GM_1$'s pentasaccharide in solid-phase assays and CG-16's capacity as growth regulator for neuroblastoma cells is reduced, highlighting this difference (in preparation). Evidently, common cross-linking properties will not necessarily translate into identical responses, a noteworthy result for further studies with other proto-type and also tandem-repeat-type galectins.

The versatility of the selected test system is illustrated by the presented analysis of galectin effects on neuronal development (neuritogenesis, axon outgrowth, guidance and regeneration). We can envisage that the effects of lectins on other aspects can be easily analysed in this system (polarization, axon branching, dendritic development or synaptogenesis). These experiments open the clinical perspective to collect data for the design of a new class of carbohydrate-binding tools aimed at ameliorating conditions affecting neuronal development and degeneration, and encourage systematic monitoring with mammalian galectins.

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