

Published in final edited form as:

*J Neurosci Res.* 2008 March ; 86(4): 821–831. doi:10.1002/jnr.21539.

## F3/Contactin-Related Proteins in *Helix pomatia* Nervous Tissue (HCRPs): Distribution and Function in Neurite Growth and Neurotransmitter Release

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### Abstract

By using antibodies against mouse F3/contactin, we found immunologically related glycoproteins expressed in the nervous tissue of the snail *Helix pomatia*. *Helix* contactin-related proteins (HCRPs) include different molecules ranging in size from 90 to 240 kD. Clones isolated from a cDNA expression library allowed us to demonstrate that these proteins are translated from a unique 6.3-kb mRNA, suggesting that their heterogeneity depends on posttranslational processing. This is supported by the results of endoglycosidase F treatment, which indicate that the high-molecular-weight components are glycosylation variants of the 90-kD chain. In vivo and in cultures, HCRPs antibodies label neuronal soma and neurite extensions, giving the appearance of both cytoplasmic and cell surface immunostaining. On the other hand, no expression is found on nonneural tissues. Functionally, HCRPs are involved in neurite growth control and appear to modulate neurotransmitter release, as indicated by the inhibiting effects of specific antibodies on both functions. These data allow the definition of HCRPs glycoproteins as growth-promoting molecules, suggesting that they play a role in neurite development and presynaptic terminal maturation in the invertebrate nervous system.

### Keywords

axonal glycoproteins; neurite growth; neurotransmitter release; F3/contactin

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In vertebrates, axonal growth and pathfinding control depend on mechanisms involving a large set of neuronal surface glycoproteins, which belong to distinct families (Dickson, 2002; De Castro, 2003; Wen and Zheng 2006; Maness and Schachner, 2007). In particular, molecules built by the association of immunoglobulin type C2 and fibronectin type III (FNIII) domains play a relevant role in basic events of neuronal differentiation as axonal growth, fasciculation, and pathfinding (Brummendorf and Lemmon, 2001), a role also played by several extracellular matrix components (Galtrey and Fawcett, 2007). Depending on the mechanism of membrane association, axonal surface glycoproteins may be classified

into two groups, i.e., transmembrane, e.g., L1/NgCAM and NrCAM (Walsh and Doherty, 1997), and glycosylphosphatidylinositol (GPI)-anchored, e.g., F3/contactin (Gennarini et al., 1989, 1991) and TAG-1 (Furley et al., 1990).

Most of these molecules display a wide species distribution, and some of them have been detected in invertebrates such as *Aplysia* and leech, where NCAM and L1 homologues, called *ApCAM* and *tractin*, have been described (Thompson et al., 1996; Xu et al., 2003). The functional role of these molecules is also likely conserved, as shown for the ApCAM ability to mediate the formation of synaptic contacts (Mayford et al., 1992) and to modulate synaptic function (Zhu et al., 1995; Schacher et al., 2000).

Based on the stereotyped organization of its neuronal connections, the nervous tissue of the land snail *Helix pomatia* represents a helpful model for elucidating the mechanisms underlying neuronal connectivity and synaptic function and for addressing in a comprehensive way the significance of adhesive glycoprotein expression in neuronal function. In this context, we have previously shown that cultured neurons from the cerebral ganglion are able to regenerate neurites and to modulate neurotransmitter release in a target-dependent manner (Ghirardi et al., 2000, 2001) and that target-bound adhesion molecules play a fundamental role in these processes (Ghirardi et al., 2001). Only few reports are available in this field (Keller and Schacher, 1990; Alenius and Bohm, 1997; Ghirardi et al., 2001), so the present study is aimed at expanding the repertoire of neuronal glycoproteins potentially involved in mediating such functions in invertebrate nervous tissues.

In particular, we focus on F3/contactin as a potentially useful molecular candidate. Originally identified in the chick (Ranscht, 1988) and mouse (Gennarini et al., 1989), F3/contactin was subsequently detected also in humans (Berglund and Ranscht, 1994), ox (Watanabe et al., 1995), rat (Shimazaki et al., 1998), fish (Haenisch et al., 2005), and *Drosophila* (Faivre-Sarrailh et al., 2004). The molecule is known to modulate several developmental events, including cell adhesion, axonal growth, pathfinding, and myelination, and is involved in synaptic function (Gennarini et al., 1991; Pesheva et al., 1993; Berglund et al., 1999; Murai et al., 2002; Bizzoca et al., 2003; Hu et al., 2003). Deletion studies indicated that such functions map to different domains within the Ig-like and FNIII-like regions (Durbec et al., 1994). Here, we use mouse F3/contactin antibodies, previously demonstrated to affect basic neuronal functions in rodents (Durbec et al., 1994; Buttiglione et al., 1996), to identify and partially to characterize related molecules expressed in *Helix* nervous tissue. The rationale for this study is to gain a broad view of the potential functional significance of this glycoprotein class through the use of a simple neuronal system.

## MATERIALS AND METHODS

### Cell Culture

The technique for culturing neurons from *Helix pomatia* has been described previously (Ghirardi et al., 1996). Briefly, C1 and C3 neurons from the cerebral ganglion and B2 from the buccal ganglion were dissociated by a thin glass micropipette, plated on poly-L-lysine (0.5 mg/ml in 0.1 M sodium tetraborate, pH 8.2; Sigma; St. Louis, MO), preincubated in *Aplysia* hemolymph for 24 hr, and cultured in L15 Leibovitz medium (Sigma) at 18°C for 3–5 days.

### Antisera

Two rabbit antisera were used: 1) the 24III serum, raised against a mouse F3/contactin- $\beta$ -galactosidase fusion protein encompassing the whole immunoglobulin region and the first FNIII repeat (Gennarini et al., 1991) and 2) the Lim antiserum, raised against a synthetic

peptide spanning the 20 N-terminal amino acids of mouse F3/contactin, coupled to keyhole limpet hemocyanin (KLH; Roche, Mannheim, Germany).

### Immunostaining Procedures

**Living cells**—Three to five days after plating, the cells were rinsed with *Helix* saline and incubated for 1 hr at room temperature (RT) with the 24III or Lim antisera [1:125 or 1:1,000, respectively, diluted in 5% bovine serum albumin (BSA) in 0.1 M phosphate-buffered saline (PBS)], using the preimmune sera at the same dilution as controls. After rinsing, TRITC-labelled goat anti-rabbit secondary antibodies were applied for 1 hr at RT. The stained cells were observed with an inverted microscope equipped with fluorescent optics, and images were acquired with an intensified CCD camera (Hamamatsu, Iwata-City, Japan), processed in Image Pro Plus (Media Cybernetics, Gleich, Germany) and stored in a Pentium IV PC.

**Fixed cells**—Alternatively, the cells were fixed with 4% paraformaldehyde in 0.1 M PBS for 40 min at RT and, after bleaching with 0.6% H<sub>2</sub>O<sub>2</sub> in ethanol, incubated in saturation buffer for 45 min at RT, followed by the above primary antisera in the presence or absence of 0.25% saponin (1 hr at RT or ON at 4°C). The 24III or Lim antisera and the corresponding preimmune sera were used at 1:125 or 1:1,000 dilutions, respectively, in saturation buffer. After a 1-hr incubation with TRITC-conjugated secondary antibodies (1:100 dilution; Jackson Laboratories; Bar Harbor, ME), labelled cells were washed and observed with fluorescence optics. Images were analyzed digitally.

### Immunohistochemistry

Immediately after dissection, cerebral ganglia were fixed by immersion in 75% picric acid containing 6.25% glutaraldehyde and 1% acetic acid, then embedded in paraffin, and 5- $\mu$ m-thick microtome sections were generated. For immunohistochemical staining, sections were first incubated with 0.3% H<sub>2</sub>O<sub>2</sub> in PBS to counteract endogenous peroxidases and then with blocking buffer [3% BSA + 5% fetal calf serum (FCS) in PBS] for 30 min. Primary antibodies (1:1,000 dilution in blocking buffer) were then applied ON at 4°C, and, after rinsing with PBS, the sections were incubated for 1 hr at RT with biotinylated goat anti-rabbit IgG (2  $\mu$ g/ml in blocking buffer; Vector, Burlingame, CA), followed by streptavidin-peroxidase (Vector; 5  $\mu$ g/ml in blocking buffer). Finally, the sections were treated with 3-amino-9-ethylcarbazole (ABC kit; Vector) for 10 min at RT. Pictures were taken with a Spot RTKE camera (Diagnostic Instruments; Sterling Heights, MI).

### Western Blot

Extracts from either mouse brain or whole *Helix* ganglia were obtained by direct suspension of the ganglia in 5 volumes of Laemmli electrophoresis sample buffer, boiled for 2 min, and then centrifuged at the highest speed in a microcentrifuge to remove genomic DNA and insoluble debris. Alternatively, tissues were homogenized in 5 volumes of RIPA buffer [50 mM Tris/HCl, pH 7.4, containing 150 mM NaCl, 0.1% NP40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 5 mM iodoacetamide, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 U/ml aprotinin, 5  $\mu$ M pepstatin, 40  $\mu$ M leupeptin, 5  $\mu$ g/ml  $\alpha$ 2-macroglobulin (Roche, Mannheim, Germany) as protease inhibitors]. After 10 min of incubation on ice, the homogenate was centrifuged (highest speed in a microcentrifuge at 4°C) to remove debris and nuclei, and the supernatant was boiled for 2 min in an equal volume of Laemmli electrophoresis sample buffer. Samples containing 100  $\mu$ g protein were separated on a 7% polyacrylamide gel transferred to nylon membranes and probed with the primary antisera (24III and Lim 1:1,000 dilution) or a rabbit preimmune serum (same

dilution), followed by horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG and detected by the chemiluminescent ECL system (GE Healthcare, Uppsala, Sweden).

### N-Glycosidase F Digestion

Tissues homogenized in RIPA buffer as above were subjected to N-deglycosylation with N-glycosidase F (Roche) as described by Gennarini et al. (1989). For controls, duplicate aliquots of the extracts were incubated in the absence of the enzyme. Equivalent protein amounts from digested and undigested samples were then analyzed by Western blotting by using the above antisera.

### Immunoaffinity Purification of Antibodies

Whole extracts from *Helix* ganglia (about 5 mg protein) were separated on 7% polyacrylamide gels and blotted onto nitrocellulose. After location of F3/contactin antibody-reactive bands, the corresponding nitrocellulose region was dissected out, saturated for 30 min at 37°C and incubated with F3/contactin antisera (1:500 dilution in saturation buffer) ON at 4°C. After several washes, bound antibodies were eluted in a 0.2 M glycine buffer, pH 2.8, and immediately neutralized with unbuffered 1 M Tris. Finally, BSA was added to the eluate to a 10% final concentration.

### Measurements of Neurite Length

To measure neurite length, phase-contrast images of the cultures were acquired in Image Pro Plus with a Sony videocamera. Measurement of neurite length was made on extensions originating from the axonal stumps. For this, a circumferential line centered at the tip of the stump and interconnecting the tips of the three longest neurites was drawn in a semiautomatic way on the computer screen in order to demarcate the area covered by the growing neurites. The radius of such a circumferential line was then taken as a measure of the extent of neurite growth. Measures were made in the presence of the 24III anti-mouse F3/contactin or the corresponding preimmune (taken as control) serum. Statistical analysis was performed using the two-way repeated-measures ANOVA, followed by multiple-comparisons Newman Keuls test.

### Detection of Neurotransmitter Release

The secretory properties of the C1 terminals were analyzed by adding a freshly dissociated assay neuron B2 to 3-5-day-old C1–C3 cocultures. The assay neuron was impaled with a glass micropipette filled with 2.5 M KCl (10–15 M $\Omega$ ) connected to an Axoclamp 2A preamplifier (Axon Instruments, Union City, CA) in bridge mode. This neuron was micromanipulated in close contact with C1 neurites, and left in situ throughout the experiment. Neurotransmitter release was evoked by electrical stimulation of C1 (10 Hz for 10 sec) every 5 min and measured as the amount of depolarization recorded in the B2 assay neuron, previously hyperpolarized 30 mV below its resting potential. The results were expressed as means  $\pm$  SEM, and statistical significance was determined by a two-way ANOVA followed by a post hoc test of Newman Keuls for multiple comparisons.

### Isolation of HCRP cDNAs From a Lambda Expression Library

A custom cDNA  $\lambda$ TriplEx2 expression library from *Helix* ganglia mRNA by oligo(dT) priming (Clontech, Mountain View, CA) was screened by using the 24III serum previously adsorbed on *Escherichia coli* lysates to counteract the background. After isolation, positive phages were converted in pTriplEx2 plasmids by *cre-LoxP* recombination mediated at *LoxP* sites of  $\lambda$ TriplEx2 and sequenced by using both 5'- $\lambda$ TriplEx2 and T7 sequencing primers at the MWG Biotech (Ebersberg, Germany) and then analyzed by using the BLAST program on the NCBI web pages (<http://www.ncbi.nlm.nih.gov/BLAST>).

## RNA Preparation and Analysis

After dissection, mouse brain and *Helix* ganglia were either immediately used for RNA preparation or frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use. Total RNA was prepared by tissue homogenization in Trizol reagent (Invitrogen, Milan, Italy) according to the manufacturer's instructions. For poly(A)<sup>+</sup> preparation, the Message Maker mRNA isolation system (Invitrogen) was used. For Northern blotting, 16  $\mu\text{g}$  of total or 2  $\mu\text{g}$  of poly(A)<sup>+</sup> RNA was separated on formaldehyde-containing 0.8% agarose gels, transferred to nylon membranes (Hybond C; GE Healthcare), and hybridized to the 960-bp insert from the isolated 9.1 clone using standard protocols (Gennarini et al., 1989).

## RESULTS

### Mouse F3/Contactin-Related Immunoreactivity Is Observed on Sections From *Helix* Ganglia

To look for mouse F3/contactin antibodies-reacting molecules expressed in *Helix* nervous tissue, sections from paraffin-embedded ganglia were processed with the 24III antiserum (Gennarini et al., 1991). In cerebral ganglia, this serum decorated the perikarya of neuronal cells and their axonal extensions (arrows in Fig. 1A,B), mostly giving the appearance of a cytoplasmic immunostaining (Fig. 1A–C). However, labelling of cell membranes, including the axolemma, was also observed (arrow in Fig. 1B). On the other hand, no labelling was observed within the nuclei nor when the preimmune serum was used (not shown), both findings supporting the specificity of the immunostaining profile. A variable labelling intensity was observed within different cells belonging to the same ganglion, strongly expressing cells being flanked by cells bearing lower amounts of the antigen (Fig. 1A). In some instances, on the perikarya, the immunostaining was concentrated on adjacent cells membranes (black arrows in Fig. 1D), and heavily stained granules were detected in the cytoplasm (Fig. 1D, white arrowheads), indicating that the underlying antigen was associated with intracellular organelles, besides being expressed at the cell membrane and within the neuronal cytoplasm.

As for the perikarya, also in the neuropil the distribution of the immunostaining was not homogeneous (Fig. 1A), strongly expressing fibers being flanked by fibers expressing lower amounts of the antigens (Fig. 1E,F). Lower expression in the trophosphonium, the domain of nerve cells where glial cells contact axons (arrow in Fig. 1C), supported the neuronal derivation of the immunoreactive molecules.

### Different F3/Contactin-Related Proteins Might Be Identified by Western Blots on *Helix* Ganglia Extracts

To identify the molecular components underlying F3/contactin immunoreactivity, extracts from *Helix* ganglia were probed by Western blot with the 24III antiserum, using a mouse brain extract as the positive control (Fig. 2). Although the typical 135-kD band was detected in mouse brain, two main proteins of 240- and 200-kD were identified in *Helix* ganglia, together with a lower Mr component around 90 kD, which was inconsistently detected in *Helix* ganglia sample buffer extracts (Fig. 2). Comparable results were obtained irrespective of whether the whole F3/contactin antiserum or antibodies immunopurified from the higher MW bands were used. No protein band was detected when the 24III preimmune serum was used (not shown). Although the 90-, 200-, and 240-kD chains were identified by antibodies against mouse F3/contactin, none of them corresponded in size to it. Therefore, we called them *Helix contactin-related proteins* (HCRPs).

## Cloning of HCRP cDNA and Identification of Its mRNA

The identification of three different HCRP protein bands raised the question of their derivation. They could arise from transcription of different genes or, alternatively, by the posttranscriptional processing of a pre-mRNA arising from a single gene. In both cases, the different isoforms should represent the translation products of different mRNAs. A further possibility, however, was that the observed protein heterogeneity arose from posttranslational processing, which would predict a single mRNA chain. To address these possibilities, we isolated HCRP-cDNA clones to identify the corresponding mRNA by Northern hybridization. For this, mouse F3/contactin antibodies were used for isolating HCRP-related cDNAs from a *Helix* nervous tissue expression library (see Materials and Methods). Of three independent clones, we focused on 9.1 cDNA, containing a 960-bp insert whose sequence is shown in Figure 3A. This cDNA was used in turn to probe Northern blots of total or poly(A)<sup>+</sup> RNA prepared from the whole ganglia. As shown in Figure 3B, a 6.3-kb band was identified by this means in both cases, indicating that the different HCRP isoforms most likely arise from posttranslational processing of a single protein backbone.

To verify whether differences in glycosylation could account for HCRP heterogeneity, extracts from *Helix* ganglia in RIPA buffer were treated with N-glycosidase F, which cleaves N-linked sugars, and probed with the 24III serum by Western blotting. As shown in Figure 3C, under these conditions, the 240-kD band could not be detected, and the 200-kD one was much fainter than in controls. On the other hand, the 90-kD isoform appeared to be predominant and composed of a doublet of closely spaced bands. These data suggested that the higher Mr HCRP isoforms arose by differential N-glycosylation of the 90-kD chain, which therefore was likely to represent the main HCRP protein backbone.

## Mouse F3/Contactin Antibodies Label the Surface of *Helix* Neurons in Culture

These data indicated that HCRPs were glycoprotein components associated with cytoplasmic compartments and intracellular organelles of *Helix* neurons and also suggested expression at the membrane level. To verify whether HCRPs were exported at the cell surface, living cultures of *Helix* neurons were immunostained with the 24III and the Lim antisera. At the same time, both sera were used on fixed cells. A couple of days after plating, *Helix* neurons developed an extensive neurite network from the initial axonal stump, and incubation with F3/contactin antibodies labelled the main axons and the branching neurites (Fig. 4A–C). A strong signal was observed on the growth cones and on the varicosity swellings (Fig. 4D–F, arrowheads). Patchy distribution of the immunostaining along the neurites indicated clustering of immunoreactive molecules. However, some distinct regions appeared immunonegative along the labelled axons (arrows in Fig. 4D–F). On living cells, similar results were obtained (Fig. 4G–I).

Together, these data indicated HCRPs expression both within the neuronal cytoplasm and at the cell surface of *Helix* neurons. The demonstration that antibodies against mouse F3/contactin recognized HCRPs in living cells allowed us to use them for functional studies.

## Functional Characterization of the HCRP Glycoproteins

We next wanted to address the potential significance of HCRPs expression in neurite growth control and in neurotransmitter release modulation in identified neurons from *Helix pomatia* ganglia (Ghirardi et al., 2001).

**Role of HCRPs in neurite growth**—To check the involvement of HCRPs in neurite growth control, cultures of *Helix* neurons plated with their initial axonal segment were used. Neurons were left to adhere to the substrate for 6 hr, when a large growth cone formed from the tip of the axonal stump. Then, the 24III (Fig. 5A) or preimmune (Fig. 5B) sera were

added to the medium (time 0), and axonal growth was measured over 48 hr. As shown in Figure 5C, measurements of neurite elongation at 24 hr indicated that, in the presence of the F3/contactin antibodies, neurite growth was only  $41.285\% \pm 5.3\%$  compared with control preparations treated with preimmune serum ( $P < 0.001$ ,  $n = 6$ ; Newman Keuls multiple-comparisons test). In the antibody-treated preparations, the neurite elongation stopped, and retraction with formation of thick neurites occurred at the 48-hr time point (Fig. 5A). In fact, quantitative analysis indicated that neurite elongation was reduced to  $17.63\% \pm 5.69\%$  compared with the value measured in control cultures at 24 hr, whereas the value of  $149.94\% \pm 8.416\%$  was measured in control neurons ( $P < 0.001$ ; Fig. 5B,C). These data showed that neurite elongation of *Helix* neurons was affected by treatment with F3/contactin antibodies.

**Involvement of HCRPs in modulation of neurotransmitter release**—We previously found that the secretory capabilities of the presynaptic C1 neuron were markedly reduced when it was cocultured with an inappropriate target such as the C3 neuron (Ghirardi et al., 2000, 2001). On the other hand, when the appropriate target B2 was inserted by micromanipulation within the network made by intermingled C3 and C1 neurites, the transmitter release from C1 increased within 30 min to the level observed in these neurons cultured in the presence of the appropriate target. By using such a model, we wanted to verify whether the positive effect of the appropriate B2 target on neurotransmitter release could be, at least in part, mediated by HCRPs expressed by these neurons. To this aim, the B2 neuron was incubated with either the 24III (1:125 dilution) or the Lim (1:1,000 dilution) antisera for 2 hr before being added to C1–C3 cocultures. Then, the amplitude of the B2 depolarization evoked by C1 electrical stimulation was measured. Immediately after the addition of either the 24III or the Lim sera-treated B2 neurons, the mean evoked depolarization was of  $6.33 \text{ mV} (\pm 0.33 \text{ mV}, n = 6)$  and  $7.5 \text{ mV} (\pm 1.8 \text{ mV}, n = 6)$ , respectively. These values did not differ from those obtained with untreated ( $4.9 \pm 2.2 \text{ mV}, n = 5$ ) or preimmune-serum-treated ( $5.1 \pm 1.1 \text{ mV}, n = 5$ ) B2 neurons (Fig. 6). However, at the next time points, although a progressive increase in the evoked depolarisation was observed in untreated ( $14 \pm 3.03 \text{ mV}$  at 20 min and  $13.6 \pm 2.04 \text{ mV}$  after 30 min) and in preimmune-serum-treated ( $17.1 \pm 8.2 \text{ mV}$  at 20 min and  $21.4 \pm 7.2 \text{ mV}$  after 30 min) B2 cells, a significant decrease was found in 24III antibody treated B2 cells at 20 min ( $4.7 \pm 0.55 \text{ mV}$ ) and at 30 min ( $5.08 \pm 0.35 \text{ mV}$ ;  $P < 0.01$  and  $P < 0.001$ ) compared with the preimmune-serum-treated cells, respectively (Newman Keuls multiple-comparisons test). Similar results were obtained at the same time points in Lim antibody-treated cells ( $3.05 \pm 1.0 \text{ mV}$  at 20 min, and  $2.88 \pm 1.29 \text{ mV}$  after 30 min of contact,  $P < 0.01$  compared with the control conditions). The above effects were unlikely to arise from a reduced responsivity of B2 to 5-HT, insofar as neither 24III nor Lim antibodies interfered with the response of B2 to bath application of exogenous serotonin ( $10^{-5} \text{ M}$ ; data not shown). Together, the functional studies indicated the HCRPs involvement in both axonal growth control and neurotransmitter release.

## DISCUSSION

Here, we report on the distribution and function of F3/contactin immunologically related glycoproteins (HCRPs) expressed in the nervous tissue of the snail *Helix pomatia*, showing that they are involved in neurite growth and modulation of neurotransmitter release.

### HCRP Structural Properties

HCRPs include three main 240-, 200-, and 90-kD glycoproteins, which differ in their sugar content. Indeed, after enzymatic N-deglycosylation, the 240-kD component disappears, whereas the 200-kD chain becomes much thinner, suggesting that these chains differ by

their content in N-linked sugars. On the other hand, the 90-kD band becomes predominant in the deglycosylated sample, which suggests that it could represent the main HCRPs deglycosylated form from which the 200-kD and 240-kD chains arise by differential addition of N-linked sugars.

Further posttranslational processing may be responsible for this protein appearing as a doublet both in native and in N-deglycosylated extracts. The existence of a unique HCRP protein backbone is consistent with the demonstration of a unique HCRP mRNA.

Based on the large carbohydrate content, HCRPs could represent nervous tissue proteoglycans. This is supported by their similarity in cellular distribution to known nervous tissue proteoglycans such as receptor protein tyrosine phosphatase (RPTP) beta and its phosphacan derivative (Garwood et al., 2003; Hayashi et al., 2005). Indeed, HCRP expression recalls most the observations for the above-mentioned vertebrate RPTP family components, being found within the cytoplasm and in particular within the Golgi complex, which suggests association with organelles (Hayashi et al., 2005). As for their cellular distribution, although RPTP beta and phosphacan were originally identified on glia (Engel et al., 1996); they are also expressed on neurons (Snyder et al., 1996; Hayashi et al., 2005). Similarly to HCRPs, the RPTP family includes isoforms arising from addition of complex carbohydrate chains to the protein backbone (Barnea et al., 1994; Maeda et al., 1994; Maurel et al., 1994). In vertebrates, such proteoglycans interact with known adhesion receptors such as NCAM, NgCAM/L1, NrCAM, and TAG-1 (Milev et al., 1996); with contactin itself (Peles et al., 1995); and with some of its ECM receptors, such as tenascin C (Grumet et al., 1994) and tenascin R (Milev et al., 1998). These molecular complexes are thought to be of key relevance for early neural developmental events, including neuronal migration (Ohyama et al., 2004) as well as axonal growth (Friedlander et al., 1994; Milev et al., 1994; Maeda and Noda, 1996).

### HCRP Distribution in Helix Nervous Tissue

Immunohistochemical staining revealed high HCRP expression within the neuronal soma and in neurite extensions. Expression in the former may correspond to forms transiting through the trans-Golgi secretory pathways, which may be relevant both for protein glycosylation and for export toward the growing axon, where HCRPs were found to be expressed. This suggests that the molecules could undergo axonal transport and, therefore, that there is a large HCRP intracellular pool, which feeds the axonally expressed components.

Within the same ganglion, HCRP expression varies from cell to cell, indicating differential regulation within different neurons. However, a heterogeneity in the staining pattern is also observed within the same cell along the neurites, resulting in discrete segments lacking these molecules. Such expression profile could depend on either regulated changes in the rate of axonal transport or on selective interactions of the molecules with components expressed along specific axonal segments as shown for interactions of the Sema 3A semaphorin component with proteoglycan receptors (De Wit et al., 2005). Finally, the demonstrated HCRP expression at the level of the axonal branching points could support a potential role for HCRPs in directional axonal growth, as demonstrated also for other vertebrates glycoprotein components such as Nogo (Richard et al., 2005).

### HCRP Functional Role

To address the functional role of HCRPs, we first explored the possibility that HCRPs mediate the control of neurite growth, in which both F3/contactin and brain proteoglycans are involved in vertebrates (Gennarini et al., 1991; Margolis et al., 1996; Margolis and



Margolis, 1997; Bizzoca et al., 2003; Schwartz and Domowicz, 2004). Indeed, neurite growth from *Helix* neurons was strongly inhibited by mouse F3/contactin antibodies, as shown in the case of several vertebrate neural adhesive molecules (see, e.g., Jones et al., 2003). Our data also provide indications that HCRPs could be involved in modulation of neurotransmitter release, which suggests complex interactions with the nerve cell signalling machinery responsible for modulating exocytosis (Rosenmund et al., 2003). In this respect, a role has been also proposed for extracellular matrix components such as the astrocyte-derived thrombospondins, involved in maintaining adhesion of pre- and postsynaptic specializations (Ullian et al., 2001; Washbourne et al., 2004; Ziv and Garner, 2004).

In conclusion, by using antibodies against mouse F3/contactin, we have found a set of glycoproteins (HCRPs) that share its role in neurite growth control in *Helix pomatia* nervous tissue. In addition, the use of a simple neuronal system has allowed us to propose a role for these glycoproteins in the regulation of exocytotic events that preside over neurotransmitter release.

## Acknowledgments

The technical assistance of Domenico Buonsanti, Dr. Claudio Franchino, and Petra Kieslinger is acknowledged. Dr. L. De Benedictis is thanked for help with cDNA cloning techniques.

Contract grant sponsor: Italian Ministry of University FIRB; Contract grant number: NE01WY7P; Contract grant sponsor: COFIN; Contract grant number: 2005055095; Contract grant sponsor: Fondazione Pierfranco e Luisa Mariani; Contract grant sponsor: Telethon Italy; Contract grant number: 1101; Contract grant sponsor: Austrian Science Foundation; Contract grant number: P17874B05.

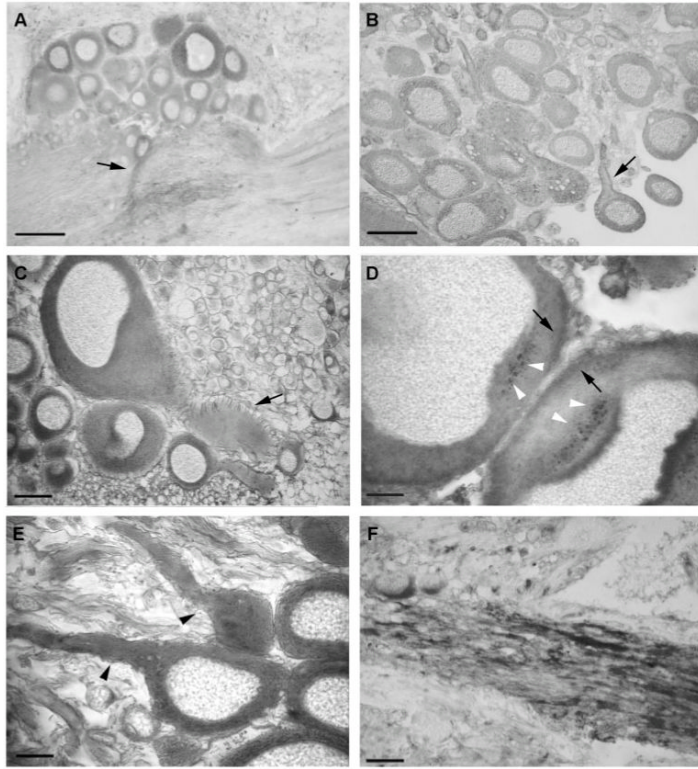
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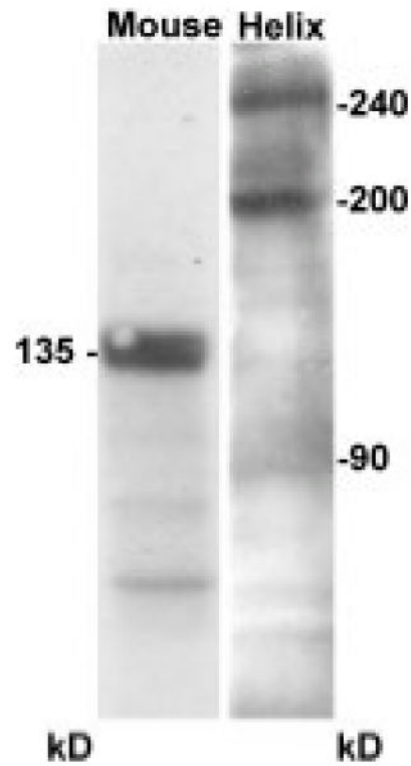
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**Fig. 1.**

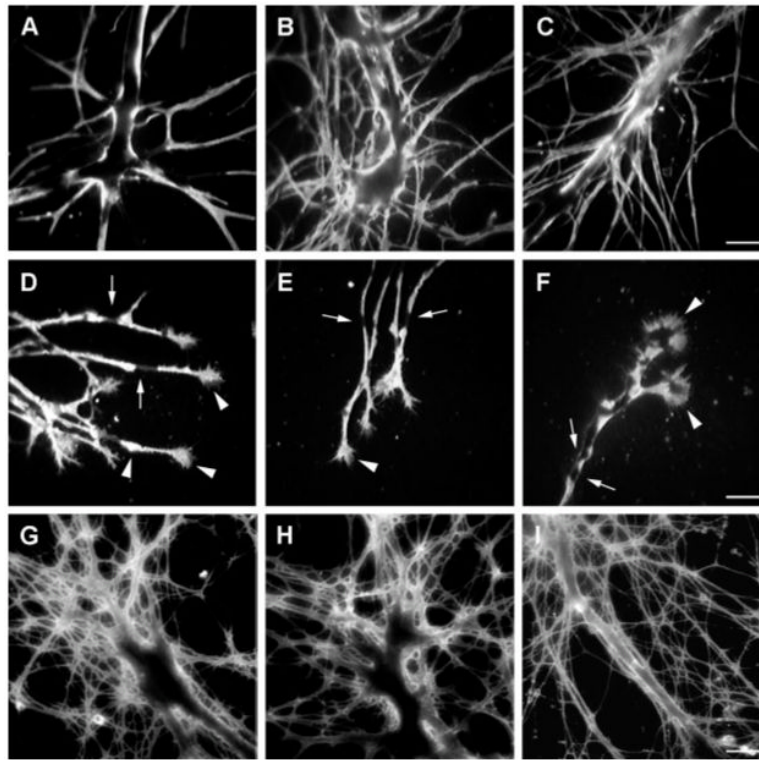
**A–F:** F3/contactin-related immunoreactivity on sections from *Helix pomatia* cerebral ganglia. Low-power pictures (A,B) demonstrated variable but relevant expression on both cell bodies and fiber tracts. Expression was observed on neurites (arrows in A,B) with both axoplasmatic and membrane (arrow in B) staining. High-power pictures (C–E) confirmed strong expression within the cytoplasm, in particular on intracellular organelles (D, white arrowheads), whereas low or absent antigen location was found within the nuclei (C–E). Antigen expression was also associated with fibers, on which a nonhomogeneous distribution was observed (A,E,F). In E, arrowheads point to low- and high-antigen-expressing axons. No expression was observed within the trophospongium (arrow in C). Scale bars = 100  $\mu\text{m}$  in A,B; 20  $\mu\text{m}$  in C,F; 10  $\mu\text{m}$  in D,E.



**Fig. 2.** Detection of F3/contactin-related molecules on *Helix pomatia* cerebral ganglia extracts. Western blots with the 24III rabbit anti-mouse F3/contactin antiserum revealed two main bands at 240 and 200 kD, together with a minor Mr component around 90 kD. The canonical 135-kD F3/contactin band was found on mouse brain extracts.

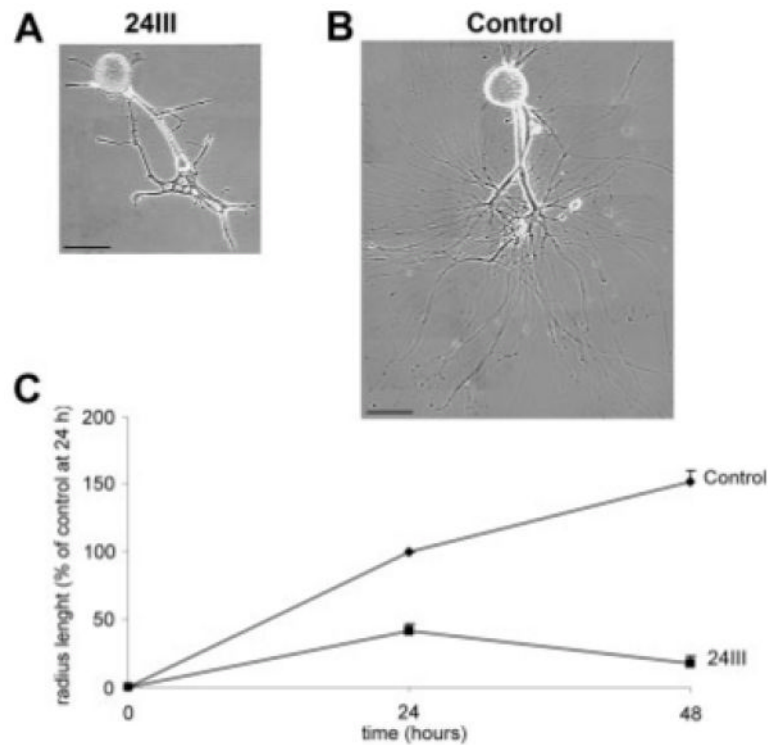


**Fig. 3.** Molecular heterogeneity of HCRPs. **A:** Isolation of a HCRP cDNA. The nucleotide sequence of clone 9.1, isolated from a *Helix* ganglia expression library with the mouse F3/contactin antiserum, is shown. **B:** Identification of the HCRP mRNA. Total and poly(A)<sup>+</sup> RNAs from *Helix* ganglia were probed by Northern blot with a <sup>32</sup>P-labelled 9.1 cDNA probe. A main 6.3-kb chain was detected in both cases. **C:** N-deglycosylation of HCRPs. Detergent extracts from *Helix pomatia* ganglia in RIPA buffer were treated with N-glycosidase F and probed with the 24III antiserum. In digested samples (+), the 240-kD band disappeared, and the 200-kD chain become thinner. Minor differences were found for the 90-kD isoform, which appeared as a doublet of closely spaced bands.

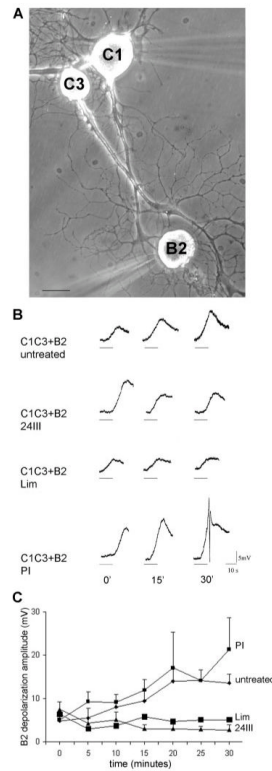


**Fig. 4.** HCRP-related immunoreactivity on cultured neurons from the cerebral ganglia. **A–F:** On fixed cells, F3/contactin antibodies stained both the main axonal stump (A–C) and the arising branching neurites. A membrane-like profile and a patchy distribution of the immunostaining were observed, indicating clustering of the corresponding surface molecules; however, some distinct regions appeared immunonegative along the labelled axons (arrows). Strong expression was also found on the growth cones and varicosities along the neurites (white arrowheads in D–F). **G–I:** HCRPs-related immunoreactivity on living cultures from the cerebral ganglia. Scale bars = 50  $\mu\text{m}$  in C (applies to A–C); 50  $\mu\text{m}$  in F (applies to D–F); 50  $\mu\text{m}$  in I (applies to G–I).





**Fig. 5.** Effects of HCRPs on neurite growth. **A,B:** Neurite growth profile of cultured *Helix* neurons in the presence of the mouse F3/contactin antiserum (24III) or in the presence of the corresponding preimmune serum (control) at 48 hr. **C:** Graphic representation of neurite growth of *Helix* neurons in the presence of mouse F3/contactin antiserum (24III) or in the presence of the corresponding preimmune serum (control). Neurite elongation was followed over 48 hr and is shown at 24 and 48 hr. Each point represents the percentage mean value ( $\pm$ SEM) of radius length of a circumferential line interconnecting the tips of the three longest neurites, normalized to the control mean value measured at 24 hr (567.2  $\mu$ m). Two-way analysis of variance (repeated measures) indicates a significant effect of treatments ( $F_{1,9} = 276.93$ ,  $P < 0.001$ ) and time ( $F_{1,9} = 6.205$ ,  $P < 0.05$ ) and a significant interaction between time and treatments ( $F_{1,9} = 43.69$ ,  $P < 0.001$ ). Scale bars = 100  $\mu$ m.



**Fig. 6.** Effects of F3/contactin antibodies on neurotransmitter release from *Helix* neurons in culture. Preincubation of the appropriate target B2 neuron with either 24III or Lim antisera inhibits its inductive effect on neurotransmitter release evoked by C1 stimulation in the presence of the wrong target. **A:** Coculture of the presynaptic neuron C1 with the wrong target C3 and the assay neuron B2 (appropriate target). The freshly dissociated neuron B2 was added to the 3-day-old C1–C3 coculture at the time of recording. The C1 and B2 neurons were then impaled with microelectrodes (out of focus) for intracellular stimulation and recording. **B:** Examples of depolarization evoked in the assay neuron B2 by a 10-Hz 10-sec train of spikes elicited in C1 (horizontal bars) immediately (0), 15 min, and 30 min after B2 addition to C1–C3 coculture in different experimental conditions (untreated, in the presence of 24III or Lim antibodies, in the presence of the preimmune serum PI). **C:** Mean amplitude ( $\pm$ SEM) of the depolarization recorded in the assay neuron B2 following C1 stimulation at the indicated time points. Diamonds and small squares represent the build up of the evoked depolarization of B2 preincubated in culture medium or preimmune serum, respectively, from 0 to 30 min after establishment of contact with neurites of C1–C3 coculture. This progressive increase of the evoked depolarization does not occur when the B2 neuron has been preincubated with 24III (triangles) or Lim (large squares) antibodies for 2 hr. Analysis of variance indicates a significant effect of treatments ( $F_{3,126} = 19.78$ ,  $P < 0.001$ ) and time ( $F_{6,126} = 2.69$ ,  $P < 0.02$ ) and a significant interaction between time and treatments ( $F_{18,126} = 1.92$ ,  $P < 0.01$ ). Scale bar = 100  $\mu$ m.