

Contactin Associates with Na⁺ Channels and Increases Their Functional Expression

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Contactin (also known as F3, F11) is a surface glycoprotein that has significant homology with the $\beta 2$ subunit of voltage-gated Na⁺ channels. Contactin and Na⁺ channels can be reciprocally coimmunoprecipitated from brain homogenates, indicating association within a complex. Cells cotransfected with Na⁺ channel Na_v1.2 α and $\beta 1$ subunits and contactin have threefold to fourfold higher peak Na⁺ currents than cells with Na_v1.2 α alone, Na_v1.2/ $\beta 1$, Na_v1.2/contactin, or Na_v1.2/ $\beta 1$ / $\beta 2$. These cells also have a correspondingly higher saxitoxin binding, suggesting an increased Na⁺ channel surface membrane density. Coimmunoprecipitation of different subunits from cell lines

shows that contactin interacts specifically with the $\beta 1$ subunit. In the PNS, immunocytochemical studies show a transient colocalization of contactin and Na⁺ channels at new nodes of Ranvier forming during remyelination. In the CNS, there is a particularly high level of colocalization of Na⁺ channels and contactin at nodes both during development and in the adult. Contactin may thus significantly influence the functional expression and distribution of Na⁺ channels in neurons.

Key words: contactin; node of Ranvier; Na⁺ channel; β subunit; axon; cluster

The function of excitable cells is highly dependent on regulation of surface density and biochemical modulation of ion channels. In neurons, voltage-gated Na⁺ channels are clustered within axonal initial segments and nodes of Ranvier, in which they serve to initiate action potentials. This distribution requires control of biosynthesis, targeting to specific axonal regions, and anchoring to the cytoskeleton. Although much is known about each of these processes, important details are lacking. Mammalian Na⁺ channels are heteromultimeric structures that include the pore-forming α subunit in association with auxiliary β subunits. At present, three β subunits and one splice variant have been identified and shown to modulate expression and voltage dependence (Isom et al., 1994, 1995a,b; Kazen-Gillespie et al., 2000; Morgan et al., 2000). $\beta 1$ is noncovalently associated with α , whereas $\beta 2$ is covalently linked to the α subunit by disulfide bonds. $\beta 3$ is highly homologous to $\beta 1$. These channels, however, exist in an even larger molecular complex that may include both *cis* (axonal) and *trans* (glial) elements. It has been shown, for example, that axonal Na⁺ channels associate with ankyrin G, providing a link to cytoskeletal elements (Bennett and Lambert, 1999).

In this study, we focused on contactin as a possible member of the Na⁺ channel signaling complex. Contactin (also known as F3, F11 in various species) is a glycosyl-phosphatidylinositol (GPI)-anchored protein expressed by neurons and glia that is thought to play multiple roles in the nervous system (Ranscht et al., 1984; Ranscht, 1988; Brummendorf et al., 1989; Gennarini et al., 1989; Koch et al., 1997). We were initially drawn to this study by the structural similarity of contactin to Na⁺ channel $\beta 2$ subunits. The extracellular region of contactin includes four fibronectin type III domains and six Ig-like domains. $\beta 2$ subunits are transmembrane proteins with a single Ig-type domain in their extracellular regions. The Ig domain of $\beta 2$ has sequence homology to the third Ig domain of contactin, and the extracellular juxtamembrane regions of these proteins are also homologous (Isom et al., 1995b; Isom and Catterall, 1996). Furthermore, tenascin-R, which accumulates at nodes of Ranvier in the CNS, binds to the Ig-like domains of contactin (Pesheva et al., 1993; Xiao et al., 1996, 1997, 1998), as well as to $\beta 2$ (Srinivasan et al., 1998; Xiao et al., 1999). Contactin also interacts with receptor protein tyrosine phosphatase β , a protein that is expressed by glia, but may also be neuronal, and has been shown to modulate Na⁺ channel function through binding to α or $\beta 1$ subunits (Peles et al., 1995; Ratcliffe et al., 2000). Contactin is also linked to the localization of axonal ion channels through its association with contactin-associated protein (Caspr)/paranodin, a neurexin family protein that forms part of the axoglial junctions at paranodes (Einheber et al., 1997; Menezes et al., 1997; Peles et al., 1997; Faivre-Sarraillh et al., 2000; Rios et al., 2000) and whose expression precedes Na⁺ channel clustering in the optic nerve (Rasband et al., 1999). Thus, numerous lines of evidence indicate a role for contactin in regulating sur-

Received May 24, 2001; revised June 29, 2001; accepted July 6, 2001.

This work was supported by National Institutes of Health Grants NS17965, DK34933, MH59980, and GM07767 and National Science Foundation Grant IBN 9734462. We thank Deana Amico and Matthew Koopmann for expert technical assistance.

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face expression of Na⁺ channels. A combination of biochemical, electrophysiological, and immunolocalization experiments all point to a specific association of contactin with Na⁺ channels that can act to regulate their functional expression.

MATERIALS AND METHODS

Antibodies. Three anti-Na⁺ channel antibodies, all against the same conserved peptide antigen within the intracellular loop between domains III and IV of the α subunit, were used with similar results. These antibodies were as follows: an affinity-purified polyclonal antibody (Dugandzija-Novakovic et al., 1995); a monoclonal antibody (Rasband et al., 1999); and an anti-SP19 polyclonal antibody obtained from Alomone Labs (Jerusalem, Israel). Rabbit polyclonal antisera to an extracellular domain of β 1 (KRRSETTAETFTWTFR), " β 1_{EX}," and the cytoplasmic domain of β 2 (KCVRRKKEQKLDST) were described previously (Malhotra et al., 2000). Polyclonal antiserum to an intracellular domain of β 1 (LAITSESKENCTGVQVAE), " β 1_{IN}," was generated and affinity purified by Research Genetics (Huntsville, AL). Polyclonal anti-contactin antibodies were raised against Ig domains 1–6 and were affinity purified for immunocytochemistry (Berglund et al., 1999). Monoclonal anti-myelin associated glycoprotein (MAG) antibodies were prepared as described previously (Poltorak et al., 1987). Monoclonal anti-neurofilament and anti- β -coatamer protein (COP) antibodies were obtained from Sigma (St. Louis, MO). Secondary antibodies were purchased from Accurate Chemical and Scientific Corp. (Westbury, NY) and Molecular Probes (Eugene, OR).

Coimmunoprecipitation. Brain membranes were prepared as described previously (Isom et al., 1995b). Membranes were solubilized in 1.25% Triton X-100, and the soluble fraction was incubated overnight at 4°C with 1 μ g of primary anti- α subunit antibody. Stably transfected cell lines coexpressing contactin and Na_v1.2 α , contactin and β 2, or contactin and β 1 were grown for 24 h after confluency before harvesting with 50 mM Tris and 10 mM EDTA, pH 8.0. Cell pellets were resuspended and solubilized in 1.25% Triton X-100, and the soluble fraction was incubated for 4 hr at 4°C with 1 μ g of anti- α , anti- β 2, or anti- β 1 antibodies, respectively. Protein A Sepharose beads (50 μ l of a 1:1 suspension) were then added, and the incubation continued for 2 hr at 4°C. The beads were washed with 50 mM Tris HCl, pH 7.5, containing 0.1% Triton X-100 and protease inhibitors. Immunoprecipitated proteins were eluted from the beads with SDS-PAGE sample buffer and separated on 7.5% acrylamide SDS-PAGE gels. Proteins were transferred to nitrocellulose and probed with anti-contactin antibody (1:1000). Chemiluminescent detection of immunoreactive bands was accomplished with WestDura reagent (Pierce, Rockford, IL).

Transfection and characterization of cell lines. Chinese hamster lung (CHL)/1610 cells were obtained from the American Type Culture Collection (Manassas, VA). 1610 cells expressing type Na_v1.2 α subunits alone or α and β 1 subunits were prepared by transfection with the appropriate cDNAs using *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulfate (DOTAP) (Roche Products, Hertfordshire, UK), as described previously (West et al., 1992; Isom et al., 1995b), and were obtained as a generous gift from the laboratory of W. A. Catterall (University of Washington, Seattle, WA). These cells, originally named SNaIIA and SNaIIA β 1–16, respectively, are denoted Na_v1.2 and Na_v1.2/ β 1 here to emphasize their subunit composition. A mammalian expression vector for mouse contactin cDNA, pRc/CMV.F3, was a generous gift from Dr. Genevieve Rougon (Centre National de la Recherche Scientifique, Marseille, France). 1610 cells were transfected with 10 μ g of pRc/CMV.F3 using DOTAP as above. Na_v1.2 and Na_v1.2/ β 1 cells were cotransfected with pRc/CMV.F3 and pcDNA3.1-Zeo (Invitrogen, Carlsbad, CA) because both of these cell lines were already resistant to G418 and were selected with 400 μ g/ml G418 (Life Technologies, Gaithersburg, MD) for 1610 cells or 400 μ g/ml Zeocin (Invitrogen) plus 200 μ g/ml G418 for Na_v1.2/contactin and Na_v1.2/ β 1/contactin cells. Na_v1.2/ β 1/ β 2 cells were prepared by transfection of Na_v1.2/ β 1 cells with pcDNA3.1-Zeo(+) β 2 and were selected with 400 μ g/ml Zeocin. β 1/contactin and β 2/contactin cells were prepared by transfection with 5 μ g of pcDNA3.1-Zeo(+) β 1 or pcDNA3.1-Zeo(+) β 2, respectively, using Lipofectamine 2000 (Life Technologies) and were selected with 400 μ g/ml Zeocin plus 200 μ g/ml G418. Na_v1.2/ β 2/contactin cells were prepared by transfection of Na_v1.2/contactin cells with pcDNA3.1-Hygro(+) β 2 using Lipofectamine 2000 and were selected with 400 μ g/ml Hygromycin B (Life Technologies) plus 200 μ g/ml Zeocin plus 200 μ g/ml G418. Na_v1.2 α / β 1/ β 2/contactin cells were pre-

pared by transfection of Na_v1.2/ β 1/contactin cells with 5 μ g of pcDNA3.1-Hygro(+) β 2 using Lipofectamine 2000 and were selected with 400 μ g/ml Hygromycin B plus 200 μ g/ml Zeocin plus 200 μ g/ml G418. Drug-resistant colonies were expanded, and total RNA was prepared using Trizol reagent (Life Technologies). For Northern blot analysis, 10 μ g of each sample was separated on 1% agarose-formaldehyde gels and blotted to nylon membranes as described previously (Isom et al., 1995a). A digoxigenin-labeled (Roche Products) antisense RNA probe was prepared from *Hind*III-linearized pRc/CMV.F3 and SP6 RNA polymerase (Invitrogen), as described previously (Isom et al., 1995a). Northern blots were detected with CDP-Star (Roche Products) and exposed to Hyperfilm ECL (Amersham Pharmacia Biotech, Arlington Heights, IL) for approximately 15 min at room temperature. Clones that were positive for contactin mRNA were expanded. For Western blot analysis, proteins were separated on 7.5 or 12% polyacrylamide gels, transferred to nitrocellulose, and probed with appropriate antibodies as indicated. Detection was with WestDura chemiluminescent reagent (Pierce). For electrophysiology, microcover glasses were pretreated with UV for 4 hr, incubated with PBS containing 0.01% poly-L-lysine as described previously (Xiao et al., 1996), washed with PBS, and dried under a sterile hood. Cells were applied to the coated coverslips and cultured for 72 hr at 37°C before use.

Whole-cell patch-clamp recordings. Coverslips with plated cells were placed in a recording chamber that was perfused with oxygenated Locke's solution containing (mM): 154 NaCl, 5.6 KCl, 2 CaCl₂, 5 D-glucose, and 10 HEPES, pH 7.4. Electrodes were filled with (mM): 140 CsCl, 1 CaCl₂, 2 MgCl₂, 11 EGTA, and 10 HEPES, pH 7.2. Whole-cell recordings were made at room temperature with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). An attempt was made to select single cells of similar size and shape from the edges of each cultured cell population for recording. The investigator was blinded to the cell type. Pipette and whole-cell capacitance and series resistance were corrected using the compensation circuitry of the amplifier. Small residual capacitive transients and leak currents were subtracted using hyperpolarizing test pulses. A laboratory computer was used to generate voltage protocols and to record and analyze currents. Typically, records were made first by generating a family of test pulses from a holding potential of -70 mV. The holding potential was then hyperpolarized in -20 mV steps to remove resting Na⁺ channel inactivation, and a new family was recorded. This procedure was repeated until peak currents reached a maximum level.

Demyelination. Lysolecithin-induced demyelination was performed as described previously (Hall and Gregson, 1971; Shrager, 1988, 1989). Briefly, adult female Lewis rats were anesthetized with chloral hydrate-pentobarbital (0.35 ml per 100 gm of animal weight), and the sciatic nerve in one leg was surgically exposed. Several branches were each injected with 1 μ l of 1% lysolecithin in sterile Locke's solution using a glass micropipette broken to a tip diameter of ~ 20 μ m. The wound was closed, and the animal allowed to recover. Nine to 70 d later, the animal was killed by CO₂ asphyxiation, and the sciatic nerve was dissected.

Immunofluorescence. CHL cells transfected with various components were fixed in 4% paraformaldehyde for 10 min and blocked with 5% calf serum in Tris-buffered saline. The primary anti-contactin antibody was used at 1:500, followed by a fluorescein-tagged secondary antibody (Vector Laboratories, Burlingame, CA). Slides were viewed on a Bio-Rad (Hercules, CA) MRC 600 confocal microscope.

Sciatic nerves from demyelinated or developing animals were dissected, desheathed, and dissociated into single fibers with collagenase-dispase (3.5 mg/ml; Sigma). In most cases, axons were teased over coverslips coated with drops of Cell-Tak (Collaborative Research, Bedford, MA), and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 30 min. Alternative procedures were used as controls or tests. In some preparations, nerves were fixed before teasing; results were identical in both procedures. To test for the possibility of antibody capping of GPI-anchored proteins, some preparations were fixed in 4% paraformaldehyde for 5 min, followed by methanol for 5 min at -20°C (Harder et al., 1998). After fixation, axons were washed in 0.1 M PB, pH 7.4, for 10 min, air-dried, and permeabilized for 2 hr in 0.1 M PB containing 0.3% Triton X-100 and 10% goat serum (PBTGS). Between steps involving antibodies, preparations were washed three times for 5 min each with PBTGS. Axons were generally double-labeled, and all antibodies were diluted in PBTGS. In one series, the Triton X-100 was eliminated from all solutions. The tissue was typically first incubated overnight with the polyclonal primary antibody, followed by a secondary goat anti-rabbit antibody coupled to Alexa488 (1:500; Molecular Probes). Axons were

then exposed to the monoclonal primary antibody and labeled with an anti-mouse secondary coupled to Cy3 (1:500; Accurate Chemicals). The preparations were allowed to air-dry and were mounted on slides using an anti-fade mounting medium. Fibers were observed under a Nikon (Tokyo, Japan) Microphot fluorescence microscope fitted with a Hamamatsu (Bridgewater, NJ) C4742-95 cooled CCD video camera. Images were collected in a laboratory computer using Image-Pro (Media Cybernetics, Silver Spring, MD).

CNS preparations were examined in cryosections. For brain sections, Lewis rats were anesthetized as above and perfused with saline containing 1% heparin, followed by 4% paraformaldehyde for 10 min before dissection. Regions of the motor cortex and underlying white matter were then cut into 2 mm sections, post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 50 min, followed by 10 min in methanol at 4°C. After washing in PB, the tissue was cryoprotected successively in 20 and 30% sucrose, frozen in OCT medium (Miller), and cut in 15- μ m-thick sections. Sections were placed in 0.1 M PB, spread over gelatin-coated coverslips, and allowed to air dry. Optic nerves were dissected, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min, in methanol at 4°C for 10 min, and then treated as for brain sections. CNS sections were permeabilized and immunolabeled as for PNS tissue.

³H-Saxitoxin binding analysis. Whole-cell saturation binding analysis of each cell line was performed using a vacuum filtration method as described previously (Isom et al., 1995b) at a concentration of 5 nM ³H-saxitoxin (STX) with the addition of 10 μ M unlabeled tetrodotoxin (Calbiochem, San Diego, CA) to assess nonspecific binding. ³H-STX (28 Ci/mmol) was obtained from Amersham Pharmacia Biotech. Binding data were normalized to protein concentrations using the BCA Protein Assay kit (Pierce).

Data analysis. Data were expressed as means \pm SEM, and statistical comparisons between groups were made with Student's two-tailed *t* test.

RESULTS

Coimmunoprecipitation

A possible association between Na⁺ channels and contactin was explored with coimmunoprecipitation. Rat brain membrane preparations were solubilized in Triton X-100 and were immunoprecipitated with anti-Na⁺ channel antibodies or control antisera. Western blot analysis with an anti-contactin antibody showed that both the lysate and the anti-Na⁺ channel immunoprecipitate contained contactin (Fig. 1*a*, lanes 2, 4). Control immunoprecipitates with nonimmune IgG (lane 1) or with antibody preabsorbed with the Na⁺ channel peptide antigen (lane 3) were negative for contactin. Because equal amounts of lysate were used in lanes 2 and 4, the difference in band densities suggests that only a fraction of the contactin in brain may be associated with Na⁺ channels. The reverse coimmunoprecipitation experiment also provided evidence for association. In Figure 1*b*, membranes were precipitated with either nonimmune IgG (left) or anti-contactin antibodies (right), and the blot was probed with anti-Na⁺ channel antibodies. Thus, at least a portion of the contactin expressed in the CNS is associated with the Na⁺ channel complex.

Na⁺ currents in transfected cells

To test for a functional interdependence of Na⁺ channels and contactin, CHL/1610 cells with stable transfections of various combinations of Na⁺ channel Na_v1.2 (SNaIIA) α and β subunits were transfected with the contactin vector pRC/CMV.F3, and mRNA was analyzed in Northern blots (data not shown). Untransfected 1610 cells did not express endogenous contactin mRNA. Cell lines with a high level of expression of contactin were selected for additional experiments. Expression of contactin protein was documented by both Western blots (Fig. 1*c*) and immunocytochemistry (Fig. 1*d–g*). In the immunocytochemistry, no permeabilization reagent was used, and the label thus represents surface expression of contactin.

Figure 2 shows families of Na⁺ currents recorded under whole-

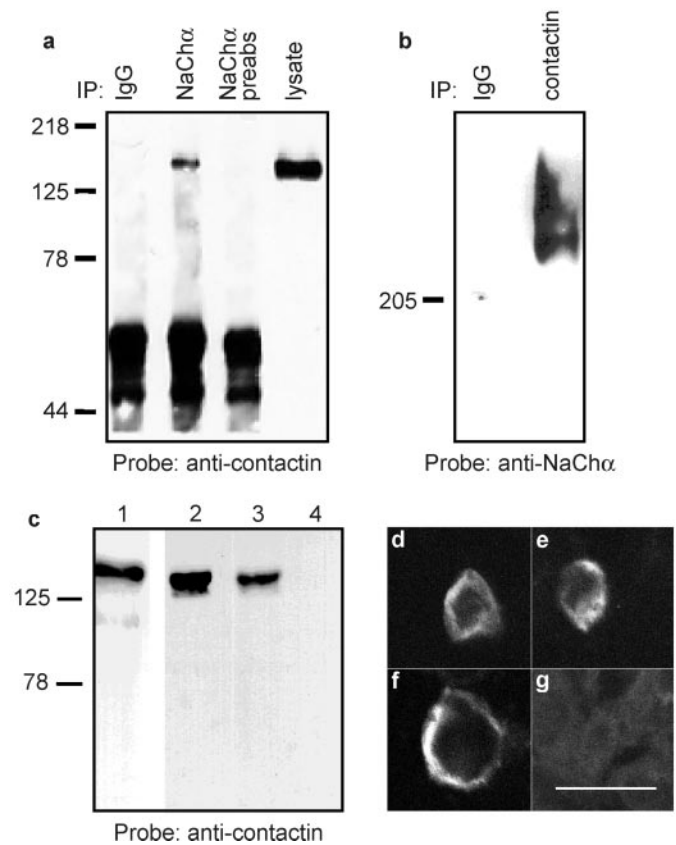


Figure 1. Association of contactin and Na⁺ channels in brain and contactin expression in transfected cells. *a*, Coimmunoprecipitation of the brain lysate with anti-Na⁺ channel antibodies, probed with anti-contactin antibodies. Lane 1, Nonimmune IgG beads as a control; lane 2, anti-Na⁺ channel (NaCh α) antibodies; lane 3, anti-Na⁺ channel antibodies preabsorbed with the peptide antigen; lane 4, lysate. The bands at \sim 135 kDa represent contactin. Equal amounts of lysate were used in lane 4 and in the coimmunoprecipitation reaction. The dark bands at lower molecular weight in lanes 1–3 represent the antibody used for immunoprecipitation. *b*, Coimmunoprecipitation of brain with anti-contactin, probed with anti-Na⁺ channel antibodies. Left lane, Nonimmune IgG; right lane, anti-contactin. *c–g*, Analysis of contactin expression in transfected cells. *c*, Western blots of cell lines probed with anti-contactin antibodies. Lanes 1 and 2, Two clones of Na_v1.2/ β 1/contactin; lane 3, Na_v1.2/contactin; lane 4, 1610 cells (untransfected). *d–g*, Immunocytochemistry of contactin expressed in transfected cells. *d*, Na_v1.2/contactin. *e*, *f*, Two clones of Na_v1.2/ β 1/contactin. *g*, Untransfected 1610 cells. Scale bar, 10 μ m.

cell patch clamp from cells with transfection combinations indicated at the left. Cells were typically held first at -70 mV, and a family of currents was recorded (Fig. 2, left column). Compared with cells transfected with Na_v1.2 α subunits alone (top records), adding either contactin or β 1 subunits individually produced little change in peak current. Adding β 2 to Na_v1.2 α / β 1 also had relatively little effect. Notably, however, peak inward currents in cells transfected with Na_v1.2/ β 1/contactin were significantly larger than those in all other cell lines. The β 2 subunit had inhibitory properties on peak currents when it was expressed together with contactin. First, Na⁺ currents were virtually eliminated in Na_v1.2 α / β 2/contactin transfected cells. Furthermore, although the Na_v1.2/ β 1/contactin cells had the largest Na⁺ currents, the addition of β 2 lowered peak I_{Na} to levels comparable with the other transfection combinations. Na⁺ current amplitude is sensitive to the level of steady-state inactivation present immediately before application of a test pulse. To judge a possible role of inactivation

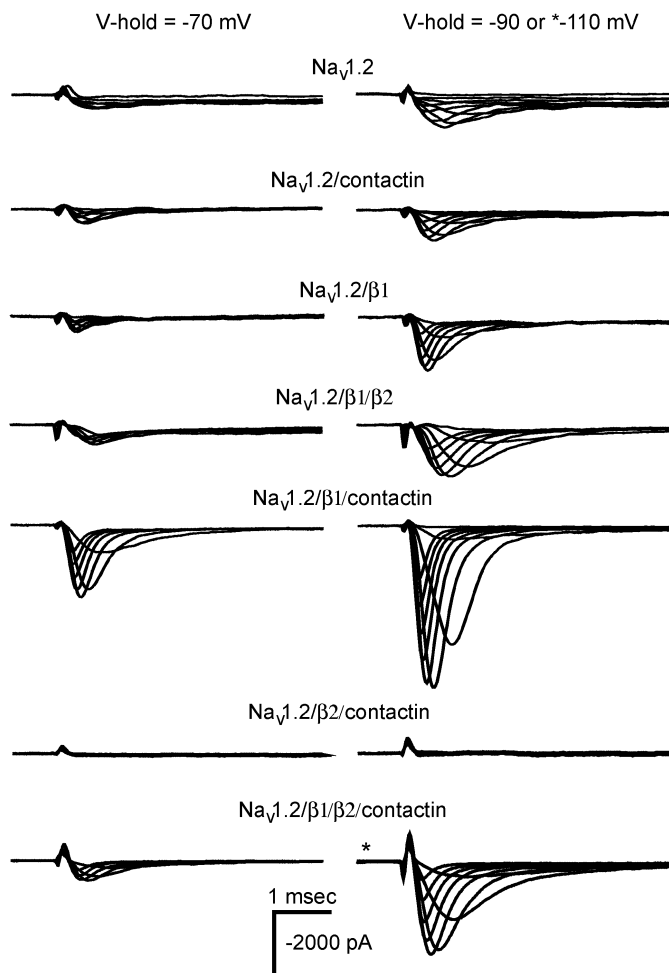


Figure 2. Na⁺ currents from transfected cells. Families of currents were recorded under whole-cell patch clamp at different HP: *left*, -70 mV; *right*, -90 mV, except for Na_v1.2/β1/β2/contactin, which were at -110 mV. Test voltages were varied in increments of 10 mV within the following ranges (chosen to bracket the maximum peak current, which was generally recorded close to 0 mV): Na_v1.2, Na_v1.2/contactin, Na_v1.2/β1/contactin, -30 to +30 (HP of -70 mV), -50 to +30 (HP of -90 mV); Na_v1.2/β2/contactin, -20 to +20 (HP of -70 mV), -20 to +20 (HP of -90 mV); Na_v1.2/β1, Na_v1.2/β1/β2, -20 to +30 (HP of -70 mV), -40 to +30 (HP of -90 mV); Na_v1.2/β1/β2/contactin, -20 to +30 (HP of -70 mV), -30 to +40 (HP of -110 mV).

in determining Na⁺ currents in the various transfected lines, especially the much larger currents in Na_v1.2/β1/contactin cells, the holding potential (HP) was hyperpolarized by 20 mV, and a new family of Na⁺ currents was recorded. This procedure was repeated until Na⁺ current amplitudes reached a maximum level, typically at HP of -90 or -110 mV. We compared first the five cell lines at the *top* of Figure 2. As seen in the *right column*, peak currents increased significantly with hyperpolarized holding potentials in all of these cells. The ratio of maximum peak inward current at the most negative holding potential applied to that at a holding potential of -70 mV was 3.0 for Na_v1.2 cells, 3.3 for Na_v1.2/contactin, 4.0 for Na_v1.2/β1, 4.6 for Na_v1.2/β1/β2, and 3.7 for Na_v1.2/β1/contactin (numbers are averages of all cells tested within each category). These numbers are all in the same range, and the ratio for Na_v1.2/β1/contactin (3.7) is identical to the average of the other four cell lines. Thus, the higher peak currents in Na_v1.2/β1/contactin cells are not attributable to a difference in resting inactivation of Na⁺ channels.

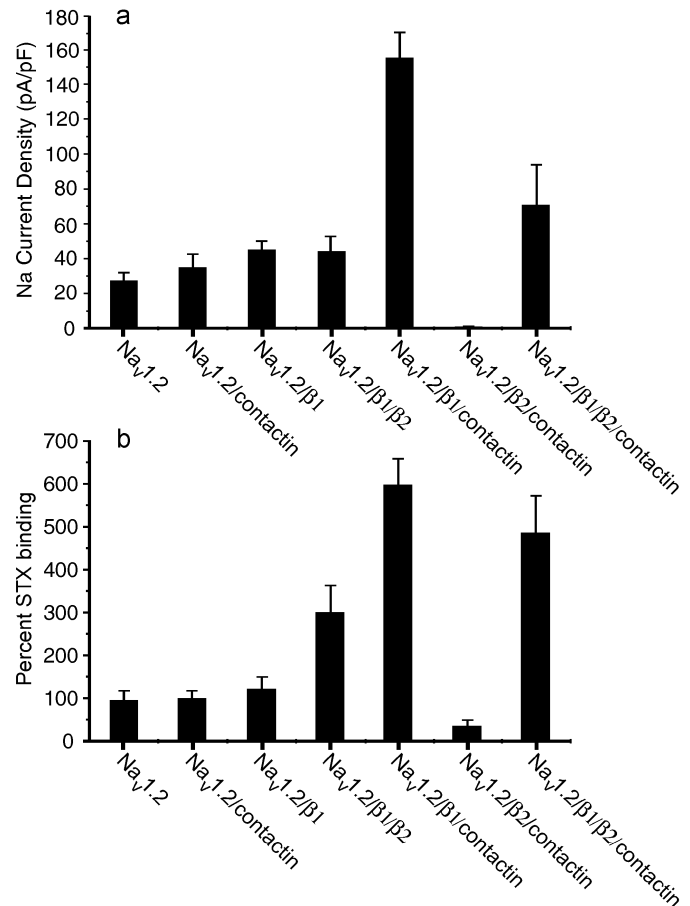


Figure 3. Na⁺ channel expression in transfected CHL cells. *a*, Maximum Na⁺ current density, plotted as picoamperes per picofaradays of cell capacitance, for the cell lines indicated along the *abscissa*. The number of cells tested for each transfection paradigm was as follows: Na_v1.2, 20; Na_v1.2/contactin, 19; Na_v1.2/β1, 30; Na_v1.2/β1/β2, 28; Na_v1.2/β1/contactin, 43; Na_v1.2/β2/contactin, 10; Na_v1.2/β1/β2/contactin, 8. The Na_v1.2/β1/contactin result included three different clones of this transfection combination. *b*, The percentage of ³H-STX binding relative to that for Na_v1.2/contactin cells, to the cell lines indicated along the *abscissa*. The data for Na_v1.2/β1/contactin, Na_v1.2/β2/contactin, and Na_v1.2/β1/β2/contactin include measurements on three different clones. In both *a* and *b*, results for Na_v1.2/β1/contactin were significantly different from those for Na_v1.2/β1 or Na_v1.2/contactin cells ($p < 0.005$).

Results for current amplitudes are summarized in Figure 3*a*, in which the mean peak Na⁺ current density for each of the cell lines tested is plotted. Hyperpolarized holding potentials were used to promote opening of all channels expressed at the surface. There was a modest increase over values with Na_v1.2 alone when β1 subunits were coexpressed, but there was a fourfold increase with the combination Na_v1.2/β1/contactin. This was not attributable to variations in cell capacitance, because the average values of this parameter for Na_v1.2, Na_v1.2/β1, Na_v1.2/contactin, and Na_v1.2/β1/contactin cells were 27, 28, 29, and 27 pF, respectively. It was also not attributable to an unusual property of one specific clone because the data in Figure 3*a* include cells from three different clones of the Na_v1.2/β1/contactin transfection group, and each alone had similarly high currents. Peak g_{Na^+} - V curves also showed a fourfold to fivefold higher maximum conductance in the Na_v1.2/β1/contactin cells than in the other lines listed above (data not shown). A difference in peak Na⁺ current could in principle result from a relief of block of expressed channels, an

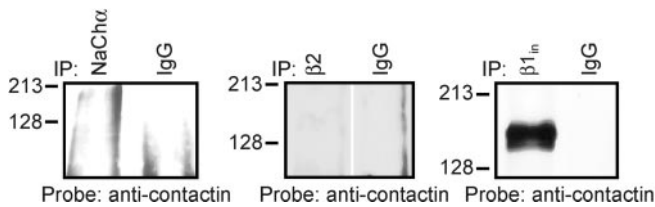


Figure 4. Association of contactin with Na⁺ channel subunits. Lysates of cells transfected with contactin plus one Na⁺ channel subunit were analyzed by coimmunoprecipitation. In each case, lanes correspond to beads with nonimmune IgG controls and antibodies, as marked. Western blots were probed with anti-contactin. *Left*, CHL/1610 cells transfected with Na_v1.2 α and contactin, immunoprecipitated (IP) with anti-Na⁺ channel α -subunit antibodies (NaCh α). *Middle*, Cells transfected with β 2 subunits and contactin, immunoprecipitated with anti- β 2. *Right*, Cells transfected with β 1 subunits and contactin, immunoprecipitated with anti- β 1_{in} antibodies.

increase in single channel conductance, or a higher density of channels in the surface membrane. As a test of the latter, we measured ³H-STX binding to these cell lines, and the results are shown in Figure 3*b*. Toxin binding for the Na_v1.2/ β 1/contactin cells was approximately fivefold higher than for Na_v1.2/ β 1 or Na_v1.2/contactin. Except for a somewhat higher relative binding level in Na_v1.2/ β 1/ β 2 cells, the ³H-STX binding data agree well with the current density measurements and argue strongly for increased expression of Na⁺ channels in the surface membrane of these cells. Again, several Na_v1.2/ β 1/contactin clones were tested, with similar results (the graph includes all cells tested).

Our work was originally stimulated by the structural homologies noted previously between contactin and the β 2 subunit. However, these proteins behaved very differently. Substituting contactin for β 2 in the presence of Na_v1.2 and β 1 increased peak currents significantly. Most interestingly, the combination Na_v1.2/ β 1/contactin produced a very high level of channel expression, whereas Na_v1.2/ β 2/contactin cells had barely detectable currents and a correspondingly low level of ³H-STX binding. Thus, there was a specific requirement for β 1 in the enhancement of Na⁺ current by contactin. Cotransfection of β 2 with Na_v1.2/ β 1 improved cell surface Na⁺ channel expression as measured by ³H-STX binding. Furthermore, adding β 2 to Na_v1.2/ β 1/contactin cells induced a strong steady-state inactivation at -70 mV, but surface density was not significantly affected. Whereas peak currents of Na_v1.2/ β 1/contactin cells were increased by a factor of 3.7 by hyperpolarized holding potentials, those of Na_v1.2/ β 1/ β 2/contactin cells increased 8.6-fold. Both Na⁺ currents and ³H-STX binding were undetectable in Na_v1.2/ β 2 cells (data not shown). Modulation by β 2 was thus dependent on coexpression of other molecules in the signaling complex.

The results suggest a unique enhancement of functional channel expression by contactin when present specifically in conjunction with β 1. We thus sought evidence for a corresponding biochemical interaction. In the coimmunoprecipitation experiments on rat brain, the Na_v1.2 α subunit of Na⁺ channels is likely to remain bound to the β 1 and β 2 subunits during immunoprecipitation with contactin, because we have used similar conditions previously to demonstrate association of α , β 1, and β 2 subunits (Malhotra et al., 2001). To identify the subunit responsible, CHL/1610 cells were transfected with contactin plus one Na⁺ channel subunit (Na_v1.2 α , β 2, or β 1). Cell lysates were prepared and immunoprecipitated with antibodies to the transfected subunit or nonimmune IgG. Western blots of the precipitates were probed with anti-contactin antibodies. Neither anti-Na_v1.2 α nor anti- β 2

subunit antibodies precipitated contactin (Fig. 4, *left, middle*). However, β 1 did associate with contactin, as seen in the band above 128 kDa in Figure 4 (*right*). No contactin was detected in the immunoprecipitate when nonimmune IgG was substituted for the antibody. Furthermore, all cell lines were tested by both Northern and Western blot to ensure that both contactin and the Na⁺ channel subunit were expressed (data not shown). Thus, three different approaches (patch clamp of I_{Na} , ³H-STX binding, and immunoprecipitation) all point to a specific interaction between β 1 subunits and contactin that results in an increased surface membrane expression of Na⁺ channels.

Immunolocalization of contactin and Na⁺ channels in PNS axons during remyelination

The spatial and temporal relationship between contactin and Na⁺ channels was explored with immunofluorescence in both the PNS and CNS. In the adult sciatic nerve, 95% of nodes had contactin label solely within paranodal regions, with none detectable in the nodal gap occupied by Na⁺ channels (Fig. 5*a*). In the remaining 5% of adult nodes, contactin was also paranodal, but with immunofluorescence extending at low levels through the region of Na⁺ channel clustering (Fig. 5*b*). After a focal intraneural injection of lyssolecithin, myelin is first vesiculated and is then removed by macrophage phagocytosis, a process that is virtually complete by 7 d postinjection (dpi) (Hall and Gregson, 1971). Over the second week, Schwann cells proliferate, adhere to demyelinated axons, and begin the process of remyelination. Schwann cells that commit to myelin formation and reach an overlapping state of ensheathment have a sharply altered pattern of protein expression, and, in particular, begin to express MAG (Martini and Schachner, 1986). Demyelinated zones become covered by short myelin segments, with new nodes of Ranvier forming at sites that were previously internodal. The cellular mechanisms involved in Na⁺ channel clustering at these new nodes have been described in detail previously (Dugandzija-Novakovic et al., 1995; Novakovic et al., 1996). Briefly, axonal Na⁺ channel clusters form just outside the tips of MAG-positive Schwann cell processes and appear to move laterally as these processes grow. Ultimately, neighboring clusters fuse to form a node of Ranvier. Immunolocalization suggests that contactin may be involved in this sequence of events. In fully demyelinated segments, before Schwann cell adherence, contactin is distributed at low density (Fig. 5*c, left*), and Na⁺ channels are likewise diffusely expressed at low levels that can be detected electrically (Shrager, 1989). At the time of initial Na⁺ channel clustering, regions of bright contactin immunofluorescence appear at the edges of many MAG-positive Schwann cell processes (Fig. 5*c, right*). At the initial stages of remyelination, as new nodes begin to form, contactin extends into the region of Na⁺ channel clustering at most sites. Figure 5*d* illustrates a single process site, with the developing paranode at the *left* and the still-demyelinated region at the *right*. Contactin extends through the zone occupied by Na⁺ channels. A quantitative analysis shows that the frequency of occurrence of this overlap is highly transient, reaching 80% at 9 dpi and then dropping to <10% at 75 dpi (Fig. 6). There are very few early sites with contactin exclusively colocalized with Na⁺ channels and none after 35 dpi. There are likewise only a small number of early nodes with contactin confined to paranodes (Figs. 5*e, 6*). Compared with remyelination, contactin is primarily paranodal during development, and contactin immunofluorescence that colocalizes with Na⁺ channels is weak (data not shown).

Figure 5. Immunofluorescence localization of Na⁺ channels (green) and contactin (red) in the PNS. *a, b*, Adult nodes of Ranvier. *a*, At ~95% of sites, contactin was below the level of detection within the nodal gap occupied by Na⁺ channels. *b*, Approximately 5% of adult nodes had a low level of contactin label colocalizing with Na⁺ channels. *c–e*, Demyelinated axons. *c*, *Left*, A demyelinated axon at 9 dpi with contactin immunofluorescence (red) widely distributed over its surface. *c*, *Right*, Early remyelination: an adherent Schwann cell labeled with anti-MAG (blue) at 9 dpi. Clusters of contactin (red) are visible at the Schwann cell edges. *d*, A single process cluster of Na⁺ channels, with the Schwann cell process at the *left*. Contactin extends into the region occupied by Na⁺ channels; 12 dpi. *e*, A new node of Ranvier at 12 dpi with contactin primarily paranodal. Scale bars, 10 μ m.

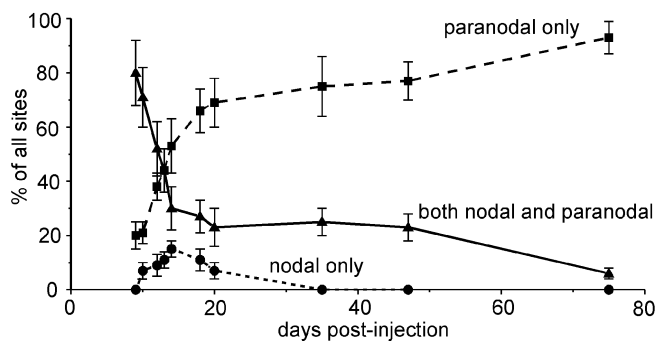
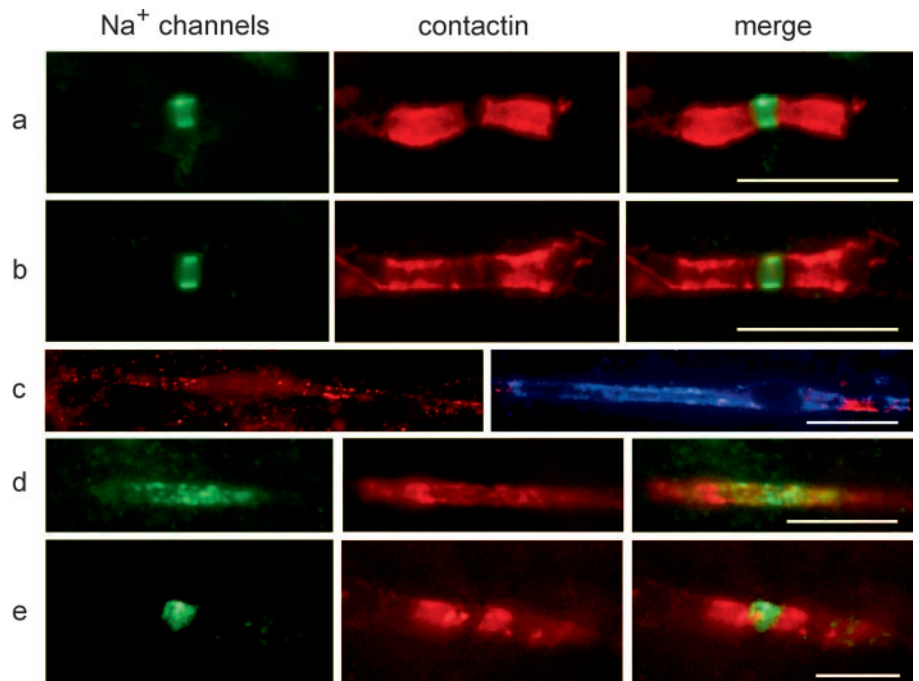


Figure 6. The frequency of occurrence of three different patterns of expression of contactin at remyelinating nodes. Sites were defined as regions containing a cluster of Na⁺ channels. *Filled squares*, Contactin only at paranodes. *Filled circles*, Contactin exclusively nodal. *Filled triangles*, contactin extending from the paranode through the nodal gap.

Immunolocalization of contactin and Na⁺ channels in the CNS

In contrast with the transient and relatively low level of colocalization in the PNS, contactin and Na⁺ channels overlapped significantly in CNS axons. Figure 7*A* illustrates axons from large cortical cells of an adult rat brain as they enter white matter. A number of nodes of Ranvier are visible (*a*), stained with the anti-Na⁺ channel antibody, and virtually all of these are also immunoreactive for contactin (*b, c*). Rat optic nerve fibers are shown in Figure 7*B*. Double-labeling for Caspr (*a*) shows that contactin is both paranodal and within the nodal gap (*b, c*). Eighty-four percent of nodal Na⁺ channel clusters were colocalized with contactin (*d–f*). At postnatal day 13, nodes in the optic nerve are at an early stage of development, and there are ~25% of the adult number of Na⁺ channel clusters visible (Rasband et al., 1999). Contactin overlaps with Na⁺ channels at these sites but is also visible in numerous small puncta (Fig. 7*C, a–c*). These foci of immunoreactivity are visible over axonal regions, which are delineated by anti-neurofilament antibodies (*d–f*) but are not seen in the intervening spaces. These gaps are presumably occupied by

glial cell bodies, because they are stained in discrete loci by an antibody to β -COP, a Golgi apparatus coat protein (data not shown).

DISCUSSION

We used a wide variety of experimental approaches, including biochemical association, electrophysiology, toxin binding, and immunocytochemistry to investigate a possible interaction of contactin with Na⁺ channels that has functional significance. Measurements were made both on cell lines engineered to express different combinations of Na⁺ channel subunits and contactin and on CNS and PNS tissue. In the cotransfection studies, we found that peak inward Na⁺ currents were significantly higher with the combination Na_v1.2/ β 1/contactin than any other group tested. What event is responsible for this result? An increase in peak current would of course result from an increased Na⁺ channel density. There are, however, several other mechanisms that could also produce higher peak currents with contactin but involve no change in channel density: a “blocking” component removed by contactin, an increase in the single channel conductance, or a change in the level of steady-state inactivation. The last of these is essentially ruled out by the experiment varying holding potential. When this voltage was changed from –70 mV to the level required for a maximum response, the peak current in Na_v1.2/ β 1/contactin cells increased 3.7-fold. This increase is virtually the same as that of the other cell types, indicating no unusual alteration in inactivation with contactin. Most importantly, ³H-STX binding paralleled the current density measurements very closely, arguing strongly for an increased surface density of Na⁺ channels as the basis for the higher currents. The toxin and electrical measurements are complimentary because STX binding alone could in principle include receptor sites on nonfunctional channels. The combined results suggest that contactin increased significantly the density of functional Na⁺ channels in the surface membrane and that the β 1 subunit was essential for this higher expression.

A possible biochemical link was explored with coimmunopre-

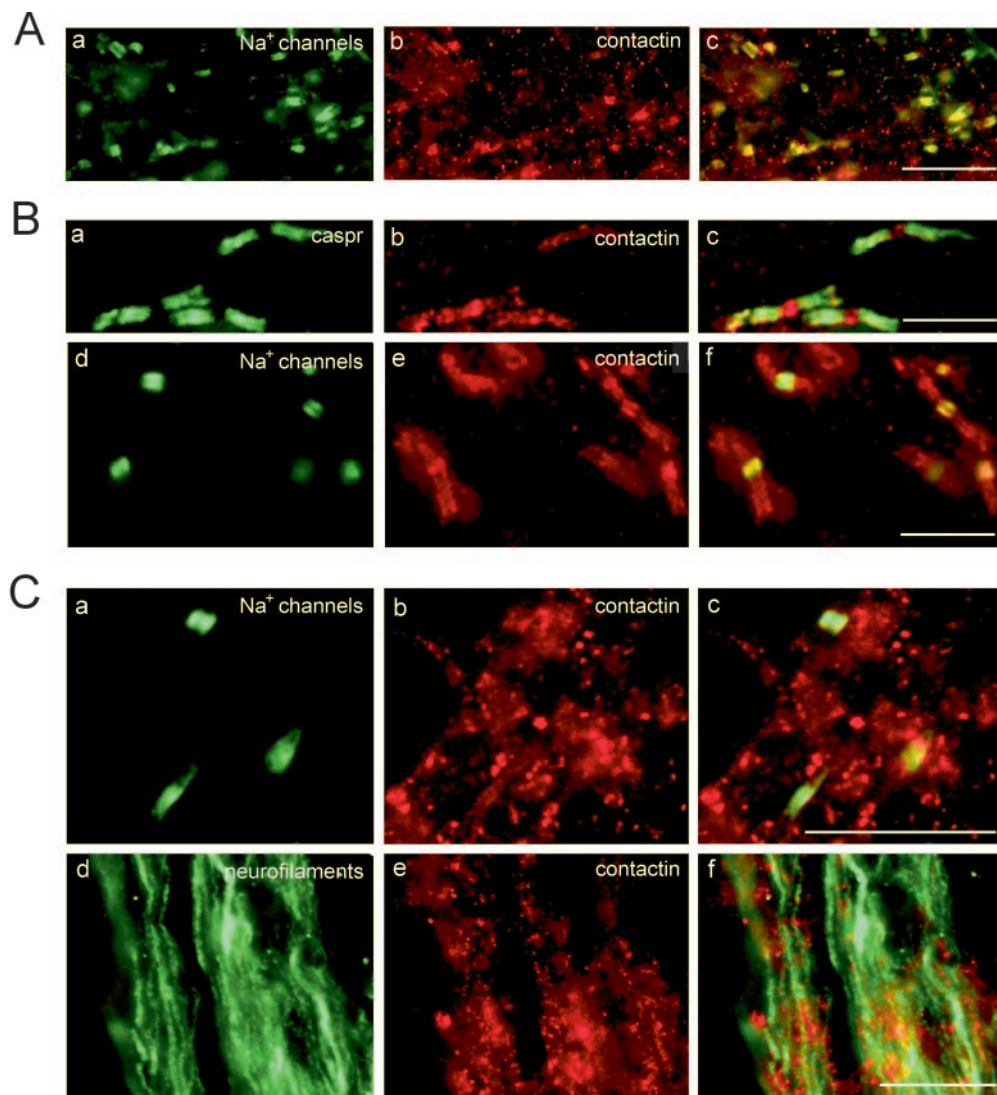


Figure 7. Colocalization of contactin and Na⁺ channels in the CNS. *Left column*, Immunolabel as indicated in each panel (*green*); *middle column*, contactin (*red*); *right column*, merge. *A*, Cryosectioned myelinated axons from large cortical cells in adult rat brain. Numerous nodes of Ranvier are visible and are stained for Na⁺ channels and contactin. Scale bar, 10 μ m. *B*, Adult rat optic nerve, double-labeled for Caspr/contactin (*a–c*) and Na⁺ channels/contactin (*d–f*). Scale bars, 5 μ m. *C*, Postnatal day 13 rat optic nerve cryosections double-labeled for Na⁺ channels/contactin (*a–c*) and neurofilaments/contactin (*d–f*). Scale bars, 10 μ m.

precipitation. Contactin and Na⁺ channels could be reciprocally immunoprecipitated from a rat brain lysate, indicating both a possible association between them and also extending the result to the CNS. The evidence suggested that only a fraction of the total contactin in the brain is associated with Na⁺ channels. This is perhaps not surprising given the many functions already known for contactin, as noted below. The immunoprecipitation experiment left open the possibility that the interaction was indirect and also did not identify which Na⁺ channel subunit is involved. We therefore examined CHL cells transfected with contactin plus just a single subunit to better define the complex and also to eliminate many possible neuronal intermediates. This experiment demonstrated that only β 1 antibodies could coprecipitate contactin. The biochemical association thus provided strong support for the results of both electrophysiology and toxin binding that pointed to the necessity for β 1 for enhancement of Na⁺ channel expression.

In neurons, Na⁺ channels are expressed at high density at axon initial segments and nodes of Ranvier. In the CNS, we found that contactin was colocalized with Na⁺ channels within the nodal gap in both the brain and optic nerve, and this association may form the basis for the copurification observed. We showed further that contactin was present at the node at 2 weeks postnatal, a time of active Na⁺ channel clustering in the optic nerve (Rasband et al.,

1999). Thus, within the CNS, we have both biochemical and morphological evidence for an interaction between contactin and Na⁺ channels. In the PNS, although contactin was only rarely seen at the node in the adult, it was transiently present at low levels early in development and, more prominently, during remyelination. As axons are remyelinated, the nodal presence of contactin is highest just as new Na⁺ channel clusters begin to form (9 dpi) and falls rapidly as the latter coalesce and become more focal.

Combining the transfection experiments that demonstrated that contactin can specifically enhance surface expression of functional Na⁺ channels with the biochemical and morphological association seen in the CNS suggests a significant role for contactin in establishing neuronal Na⁺ channel distributions. The results do not argue that contactin has a direct role in the glial-dependent Na⁺ channel clustering that accompanies formation of nodes of Ranvier. This work suggests rather that contactin may be important for the increased surface expression that would be required for subsequent glial-directed clustering. There are significant differences between nodes of Ranvier in the PNS and CNS, including the possibility that Na⁺ channel clustering is contact dependent in the former but secretion dependent in the latter (Kaplan et al., 1997). The role of contactin at these two

structures may thus also differ, with a unique requirement for stability of Na⁺ channel clusters at mature nodes in the CNS. In the PNS, participation seems to be limited to a more transient association with Na⁺ channels. Recently, we have reported also evidence for participation of β subunits in cell adhesion through interaction with extracellular matrix components and the cytoskeleton (Xiao et al., 1999; Malhotra et al., 2000). Using *Drosophila* S2 cells, it was shown that homophilic adhesion of $\beta 1$ or $\beta 2$ subunits in the presence or absence of Na_v1.2 α subunits results in cellular aggregation and recruitment of ankyrin to points of cell–cell contact. β subunit mutant constructs that lack the intracellular domains cause cellular aggregation but are incapable of recruiting ankyrin. In addition, $\beta 1$ or $\beta 2$ subunits and ankyrin G can be coimmunoprecipitated from solubilized rat brain membranes, suggesting that they may participate in a signaling complex, although additional investigation will be required to demonstrate a direct interaction.

Contactin thus appears to have multiple roles in axonal development and repair. As a GPI-anchored protein, it may be targeted to the axon through its incorporation in detergent-insoluble sphingolipid-cholesterol rafts (Olive et al., 1995; Ledesma et al., 1998), and it could require binding partners to effect intracellular signaling. Through an association with an α - $\beta 1$ complex, it could promote surface expression of functional Na⁺ channels in the axolemma, leading ultimately to high-density clusters at nodes of Ranvier. It is important in this regard to note that contactin also binds NrCAM and neurofascin, other membrane glycoproteins that are expressed at nodes and that have been implicated in Na⁺ channel clustering (Grumet, 1997; Lambert et al., 1997; Volkmer et al., 1998). Does contactin play a primary role in targeting Na⁺ channels to nodes in the CNS? On the one hand, its distribution in both nodes and paranodes would argue against this idea. However, it has been shown recently that one isoform of contactin associates closely with Caspr and colocalizes in paranodes, whereas another isoform, differing in glycosylation, is independent of Caspr and extends into the nodal gap of CNS axons (Rios et al., 2000), leaving open the possibility of some regional specificity. Alternatively, contactin may serve as a cofactor to promote high levels of functional Na⁺ channels, which then are targeted to nodes by additional mechanisms. Contactin is required for surface expression of Caspr *in vitro* (Favre-Sarrailh et al., 2000), and, through this association, it may help to stabilize the axoglial junctions as paranodes mature. In mice with contactin genetically deleted, axonal and dendritic guidance and interactions are disrupted in the cerebellum, and paranodes are abnormal (Berglund et al., 1999, 2000). Finally, since oligodendrocytes also express this molecule (Koch et al., 1997; Kramer et al., 1999), interactions with Na⁺ channels may be *cis* or *trans*, and contactin may thus mediate in part the glial-neuronal communication involved in ion channel organization.

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