Nogo-66 Receptor at Cerebellar Cortical Glia Gap Junctions in the Rat

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Key Words

Nogo · Nogo-66 receptor, rat · Glia · Gap junction · Cerebellum

Abstract

Nogo-A is a myelin inhibitor of neurite outgrowth that accounts for the difficulty in fiber regeneration in the central nervous system. Its 66-amino-acid extracellular domain (Nogo-66) contributes to the inhibitory activity of Nogo-A. The Nogo-66 receptor is widely distributed in neurons of the central nervous system, including the cerebellum. In our study on the distribution of Nogo-66 receptor in the cerebellar cortex in the rat, we unexpectedly found Nogo-66 receptor immunoreactivity in the glia cells, particularly abundant beneath the Purkinje cells. The presence of Nogo-66 receptor in glia cells has not been reported before. A detailed study was thus conducted. Immunoelectron microscopic investigation clearly demonstrated that the Nogo-66 receptor immunoreactivity could be ascertained at the gap junction between glia cells, indicating that the Nogo-66 receptor may modulate the communication between glia cells through gap junctions.

Introduction

Nogo-A is a myelin inhibitor of neurite outgrowth that accounts for the difficulty in fiber regeneration in the central nervous system [1–3]. Its 66-amino-acid extracellular domain (Nogo-66) has been reported to be one of the major domains that underlie the inhibitory activity of Nogo-A [2, 4]. Nogo-A was first found in oligodendrocytes. Later, it was found to be widely expressed in central nervous system neurons as well [5–8], indicating that Nogo-A may have a diversity of functions [9]. The Nogo-66 receptor (NgR) has an ectodomain and a glycosylphosphatidylinositol anchorage site [4]. It is widely distributed in neurons of the central nervous system, including the cerebellum [4, 7, 10, 11]. In our scrutiny for NgR immunoreactivity (irty) in the cerebellum, we found that it could be identified in the neuropil, particularly beneath the Purkinje cells. A detailed study was thus conducted to characterize the neuropil NgR-irty and found unexpectedly that the NgR-irty could be found in the glia cells, which had not been reported before. Moreover, immunoelectron microscopic study demonstrated that the NgR-irty could be localized at the glial gap junctions, indicating that NgR may modulate the activity of the gap junction.

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Fig. 1. NgR-irty in the cerebellar cortex. **A** Omission of primary antibody control. Differential interference contrast microscopy to bring out the structural background. No staining could be detected above a very faint background. **B** Note the relatively weak NgR-irty in the molecular layer (m) and the strong NgR-irty in Purkinje cell layer (p) and granule layer (g), particularly next to the Purkinje layer (see **A**). There is often a clear space surrounding the NgR-immunoreactive Purkinje cell which is shown in figure 3A to be occupied by astrocytic processes. Thus, the NgR-irty is distributed on the periphery and in the cytoplasm of the Purkinje cells. Bars = 25 μ m.

Materials and Methods

Male adult Sprague-Dawley rats (weighing 250–280 g) were used. For light microscopic immunohistochemistry, the rat was deeply anesthetized with 1% sodium pentobarbital (50 mg/kg i.p.) and perfusion fixed with 0.9% saline followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The cerebellum was removed and cryoprotected in successive 20 and 25% sucrose at 4°C for 24 h each. Sections (8 μ m) were cut on a cryostat. The sections were treated for 1 h in phosphate-buffered saline (PBS) containing 5% bovine serum albumin, 5% normal goat serum, and 0.3% Triton X-100 and incubated overnight at 4°C with the rabbit antibody against NgR (1:300; Alpha Diagnostic International, San Antonio, Tex., USA). The antibody recognizes 17 amino acids near the C terminus of NgR. The sections were incubated in biotinylated antirabbit IgG (1:400; Sigma Chemical, St. Louis, Mo., USA) at room temperature for 2 h and visualized by means of the glucose oxidase-DAB-nickel method [12]. For double immunostaining, the sections were incubated in a cocktail of anti-NgR (1:400) and the monoclonal antibody against the astrocyte marker GFAP (1:1,000; Sigma) or against the oligodendrocyte marker RIP (1:600; Chemicon International, Temecula, Calif., USA). A mixture of Alexa Fluor 488 conjugated goat antirabbit IgG (for NgR, 1:400; Molecular Probes, Eugene, Oreg., USA) and Texas Red conjugated goat anti-mouse IgG (for GFAP or RIP, 1:800; Molecular Probes) was used as secondary antibodies. An FV300 laser scanning microscope (Olympus, Tokyo, Japan) was used for confocal examinations. Omission of the primary antibodies served as negative controls.

Western blotting was performed for checking the specificity of the anti-NgR. The rat was decapitated, and the head was immediately put on ice. Cerebellum and cerebral cortex were carefully dissected out and immediately frozen in liquid nitrogen. They were then kept at –70°C before use. For protein extraction, each sample was homogenized on ice in RIPA buffer (Tris-HCl 50 mM, pH 7.5, NaCl 150 mM, NP-40 1%, sodium deoxycholate 0.5%, SDS 0.1%, and complete protease inhibitor cocktail added at a volume ratio 1:50) and centrifuged at 13,000 g at 4°C. Samples from the supernatant were loaded on 10% SDS-PAGE. The proteins were electrotransferred to a polyvinylidene difluoride membrane and reacted with anti-NgR (1:1,000) and antirabbit IgG-POD (1:12,500) and chemiluminescent solution (chemiluminescence Western blotting

kit; Boehringer Mannheim, Mannheim, Germany). Western blotting was performed according to the kit protocol.

For immunoelectron microscopy, the rats were perfusion fixed with a mixture of 4% paraformaldehyde, 0.05% glutaraldehyde, and 15% (v/v) saturated picric acid in 0.1 M phosphate buffer (pH 7.4) for 30 min. The cerebellum was removed and postfixed in the same fixative for 3 h at 4°C. Tissue sections of 50 μ m were prepared with a vibratome and placed into 0.01 M PBS (pH 7.4) containing 25% sucrose and 10% (v/v) glycerol for 1 h for cryoprotection. After being subjected to a freeze-thaw treatment, the sections were immersed in 0.01 M PBS containing 5% bovine serum albumin and 5% normal goat serum for 4 h to block nonspecific immunoreactivity. The sections were incubated with anti-NgR (1:300) for 48 h at 4°C, rinsed in 0.01 M PBS, and incubated with biotinylated goat antirabbit IgG (1:200; Sigma) followed by goat antirabbit IgG conjugated to 1.4-nm gold particles (1:100; Nanoprobes, Stony Brook, N.Y., USA) at room temperature overnight. After rinsing, the sections were postfixed with 2% glutaraldehyde in 0.01 M PBS for 45 min. Silver enhancement was performed in the dark with a HQ Silver kit (Nanoprobes). Before and after the silver enhancement, the sections were rinsed with deionized water several times. The sections were further fixed with 0.5% osmium tetroxide in 0.1 M phosphate buffer for 1 h, dehydrated with graded ethanol, replaced with propylene oxide, and flat embedded in Epon 812. The NgR-immunoreactive areas of the Purkinje and granular layers of the cerebellar cortex were selected and trimmed under a stereomicroscope and mounted onto blank resin stubs. Ultrathin sections were prepared on an LKB Nova Ultratome (LKB Instruments, Bromma, Sweden) for observation under a JEM-1230 electron microscope (JEOL, Tokyo, Japan) after being counterstained with uranyl acetate and lead citrate.

Results

Light Microscopic Study

The primary antibody omission controls showed no labeling above background (fig. 1A). Western blot of both cerebral cortex and cerebellum showed a single band at

~85 kDa, corresponding to the molecular weight of NgR (fig. 2).

Light microscopic immunohistochemistry displayed fine granular NgR-irty in all three layers of the cerebellar cortex: in the molecular layer it appeared as sparsely scattered fine granules, often lining radially, presumably along the paths of the Purkinje dendritic shafts and/or the Bergmann glia; in the Purkinje cell layer, there was a clear space surrounding each circular or spherical NgR-irty of the Purkinje cells; the granular cell layer showed strongest NgR-irty, densest next to the Purkinje cell layer (fig. 1B). It is clear from the confocal image that the space surrounding the Purkinje cells was occupied by astrocytic processes (fig. 3A). Thus, the NgR-irty in the Purkinje cell layer is distributed on the periphery and in the cytoplasm of the Purkinje cells.

Double immunostaining and confocal microscopic analysis showed that the NgR-irty occurred at either the GFAP-immunoreactive astrocytic or the RIP-immunoreactive oligodendrocytic processes (fig. 3).

Electron Microscopic Study

Very unexpectedly, under the electron microscope, NgR-irty could be identified at gap junctions which may imply a hitherto unknown functional aspect of the NgR. We, therefore, focused our ultrastructural study on the gap junction. Typically layered gap junction profiles were frequently found between glia cells. NgR-irty could be identified at gap junctions between glia cells, either between astrocyte and oligodendrocyte (fig. 4), or between astrocytes (fig. 4C). The layered profile extended for longer or shorter stretches. Clusters of silver-enhanced immunogold particles occurred predominantly along the cytoplasmic surface of the junction, seldom any could be identified in the gap. At their ends of the layered profile, the contacting cell membranes either diverged or often blurred out due to tilting of the section plane, where immunoparticles were frequently observed (fig. 4B).

Discussion

The results of the Western blot supported the specificity of the antibody we used. The NgR is anchored to the lipid raft of the cell membrane by glycosylphosphatidylinositol and is exposed to the outer surface of the membrane. In the gap junction, the NgR is located in the gap between the counterparts forming the junction. In the present study, however, seldom any silver-enhanced immunogold particles could be ascertained in the gap. Ap-

Fig. 2. Western blots. Lane 1: cerebral cortex which is known to have plenty of NgR and was used here as a positive control. Lane 2: cerebellum. In both lanes, there is a single band at ~85 kDa, corresponding to the molecular weight of NgR.

parently, this is due to the fact that the gap between the apposing membranes of the junction measures only 2–3 nm, much too narrow for IgG molecules to squeeze into [for the size of IgG, see 13]. Furthermore, the gap is tightly packed with connexin channels joining the two sides which further impede passing of immunoreagents into the gap. The layered profile of the gap junction can be seen only, if it is cut perpendicular to its plane. Tilting of the cutting angle will result in blurring out of the layers, meanwhile exposing the outer surface of the apposing membranes and the NgRs anchored on them, rendering them available for immunohistochemical reagents. Thus, in the present study, the immunogold particles were often seen in clusters at the blurred part of the gap junction

Fig. 3. Confocal microscopy of the cerebellar cortex. NgR-irty, green; GFAP-irty and RIP-irty, red. **A** NgR-/GFAP-irties. In the molecular layer, the NgR-irty often appears as radiating processes. In the Purkinje cells, the NgR-irty appears on the periphery and in the cytoplasm. The cells bodies are surrounded by astrocytic processes. Colocalization of a number of the NgR-irty dots with astrocytic processes can be seen; the arrows point to some of them as examples. The inset is a blowup of the boxed area to show more clearly the colocalization. **B** NgR-/RIP-irties. The arrows point to some of the colocalized NgR-/RIP-irties. The inset is a blowup of the boxed area. The thickness of the confocal optic section is 0.173 μm . Bars = 50 μm ; inset bars = 25 μm .

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Fig. 4. Ultrastructural localization of NgR-ir. **A** Gap junction (arrow) between an astrocyte (A), which is characterized by the presence of compact bundles of glial filaments (f), and an oligodendrocyte (O). The asterisk shows the cross section of a bundle of microtubules. Abundance of microtubules is one of the characteristics for identifying oligodendrocyte [25, p. 298]. Silver-enhanced gold particles can be seen along both sides of the gap junction. **B** Gap junction (small arrow) between an astrocyte (A) and an oligodendrocyte (O). The large arrows point to the blurred ends of the gap junction with clusters of NgR-immunoreactive immunogold

particles. f = Glial filaments. **C** Two layered profiles of a gap junction can be seen (arrowheads). At their blurred ends, clusters of gold particles are evident. G = Nucleus of a neuron of the granular layer; O = Oligodendrocyte. The inset shows the detail of the gap junctions. A1 and A2 are two veil-like astrocytic extensions, the velate astrocytes [25, p. 282]. A1 forms the junction between the astrocyte and an oligodendrocyte (O) with many microtubules; A2 forms the junction between two astrocytes. Bars in **A-C** = 200 nm; the bar in the inset of **C** = 100 nm.

profiles. The appearance of immunogold particles at the cytoplasmic side of the gap junctions occurred, apparently, because the cell membrane was made permeable to immunoreagents by freeze-thaw treatment for preembedding immunoelectron microscopy and because the epi-

tope the antibody recognizes is close to the anchoring site of the NgR on the membrane.

The gap junction has been recently found to be functionally adjustable [14–21]. It can be adjusted by phosphorylation of connexins [15, 16, 18, 19], and a blocker

has been found for opening and closing of hemichannels [20, 21]. The presence of NgR at the gap junction suggests that it may play a role in modulating the function of the gap junction. The signal transduction pathways of NgR have not been fully elucidated. The NgR-p75NTR receptor complex activates Rho which eventually results in inhibition of axonal growth; in addition, activation of the NgR-p75NTR receptor complex also activates G_i protein which prevents an increase in cAMP through inhibition of adenylate cyclase [22], and it has been reported that activation of cAMP-dependent protein kinase can lead to a rapid augmentation in connexin43 phosphorylation, resulting in an increase in the intercellular communication through the gap junction [23, 24]. Thus, there is at least one possible link between the signal transduction path-

way of the NgR and the functional modulation of the gap junction.

This paper is the first report on the presence of NgR in glia cells and at their gap junctions. The functional significance of NgR at gap junctions is certainly a very interesting and important issue to be elucidated.

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