Neurexin-1, a presynaptic adhesion molecule, localizes at the slit diaphragm of the glomerular podocytes in kidneys

Akira Saito,1,2 Naoko Miyachi,1 Taeko Hashimoto,1 Tamaki Karasawa,1,3 Gi Dong Han,1,4 Mutsumi Kayaba,1, Tomoyuki Sumi,1 Masayuki Tomita,1 Yohei Ikezumi,2 Kenji Suzuki,5 Yasushi Koitabashi,2, Fujio Shimizu,2, and Hiroshi Kawachi1

1Department of Cell Biology, Institute of Nephrology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan; 2Department of Pediatrics, St. Marianna University School of Medicine, Kawasaki, Japan; 3Department of Pediatrics, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan; 4Department of Food Science and Technology, Yeungnam University, Gyeongsan, Republic of Korea; 5Department of Gastroenterology and Hepatology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan; and 6Niigata Seiryo University, Niigata, Japan

Submitted 6 October 2009; accepted in final form 2 November 2010

Saito A, Miyachi N, Hashimoto T, Karasawa T, Han GD, Kayaba M, Sumi T, Tomita M, Ikezumi Y, Suzuki K, Koitabashi Y, Shimizu F, Kawachi H. Neurexin-1, a presynaptic adhesion molecule, localizes at the slit diaphragm of the glomerular podocytes in kidneys. Am J Physiol Regul Integr Comp Physiol 300: R340–R348, 2011. First published November 3, 2010; doi:10.1152/ajpregu.00640.2009.—The slit diaphragm connecting the adjacent foot processes of glomerular epithelial cells (podocytes) is the final barrier of the glomerular capillary wall and serves to prevent proteinuria. Podocytes are understood to be terminally differentiated cells and share some common features with neurons. Neurexin is a presynaptic adhesion molecule that plays a role in synaptic differentiation. Although neurexin has been understood to be specifically expressed in neuronal tissues, we found that neurexin was expressed in several organs. Several forms of splice variants of neurexin-1a were detected in the cerebrum, but only one form of neurexin-1a was detected in glomeruli. Immunohistochemical study showed that neurexin restrictedly expressed in the podocytes in kidneys. Dual-labeling analyses showed that neurexin was colocalized with CD2AP, an intracellular component of the slit diaphragm. Immunoprecipitation assay using glomerular lysate showed that neurexin interacted with CD2AP and CASK. These observations indicated that neurexin localized at the slit diaphragm area. The staining intensity of neurexin in podocytes was clearly lowered, and their staining pattern shifted to a more discontinuous patch pattern in the disease models showing severe proteinuria. The expression and localization of neurexin in these models altered more clearly and rapidly than that of other slit diaphragm components. We propose that neurexin is available as an early diagnostic marker to detect podocyte injury. Neurexin coincided with nephrin, a key molecule of the slit diaphragm detected in a presumptive podocyte of the developing glomeruli and in the glomeruli for which the slit diaphragm is repairing injury. These observations suggest that neurexin is involved in the formation of the slit diaphragm and the maintenance of its function. proteinuria; nephrotic syndrome; nephrin
Neurexins are reported to interact with the synaptic vesicle-associated molecules, and are postulated to play a role in synaptic vesicle docking (11, 34). It is also known that neurexins are a presynaptic transmembrane protein and have a major role in cell-cell interactions across the synapse (47). Neurexins are reported to bind CASK, a member of the membrane-associated guanylate kinase (MAGUK) family (11), via a PDZ-binding site in the intracellular domain of neurexin. Recently, CASK was reported to be expressed in podocytes and to interact with nephrin (23). These properties of neurexin prompted us to hypothesize that neurexin is expressed in podocytes and is involved in the maintenance of podocyte function. To elucidate this hypothesis, we analyzed the expression of neurexin in kidney glomeruli.

In the present study, we demonstrated that neurexin is restrictedly expressed in podocytes in kidneys and that neurexin is localized at the cytoplasmic area around the slit diaphragm. We also showed that the expression of neurexin is clearly lowered in the injured podocytes. These findings suggest that neurexin is associated with the slit diaphragm.

MATERIALS AND METHODS

Animals. Specific pathogen-free, 7-wk-old female Wistar rats (Charles River Japan, Atsugi, Japan) weighing 150–170 g were used for the induction of the experimental models. Normal rats were killed, and the organs (cerebrum, cerebellum, medulla oblongata, thymus, lung, heart, stomach, pancreas, spleen, liver, adrenal, whole kidney, colon, small intestine, uterus, ovary, placenta, testes, bladder, skin and muscle) were removed. The materials were used for immunofluorescence and RT-PCR. All animal experiments conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures for the present study were approved by the Animal Committee at Niigata University School of Medicine, and all animals were treated according to the guidelines for animal experimentation of Niigata University.

Metanephroi, cultured podocytes, human kidney sections. Metanephroi were removed from rats of 13.5, 15.5, and 18.5 embryonic days and from neonatal rats. A conditionally immortalized mouse podocyte cell line was kindly donated by Dr. Peter Mundel (Albert Einstein College of Medicine, Bronx, NY). Cultivation of the cell line was conducted as described previously (28). In brief, podocytes were maintained in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% FBS (Life Technologies, Grand Island, NY). To propagate podocytes, cells were first cultivated at 33°C with IFN-γ and maintained for 2 wk at 37°C to induce differentiation without IFN-γ. The human kidney specimen used for immunofluorescence study was isolated from the normal part of kidney tissue that had been obtained from nephrectomy of a previous study.

Experimental nephrotic models. To induce PAN nephropathy, a total of 24 rats were intravenously injected with 10 mg/100 g body wt of PAN. Six rats each were killed just before the injection 1 h, 24 h, 10 days, and 28 days after injection. Twenty-four-hour urine samples were collected on 1, 3, 5, 7, 10, 14, and 28 days after injection.

Antibody-antigen complex was washed 5 times with PBS containing 0.1% Triton X-100, and then the antigen was eluted with SDS-PAGE sample buffer. The elution fractions were separated by SDS-PAGE.

Anti-nephrin antibody (ANA)-inducing nephropathy was performed by an intravenous injection with 15 mg/rat or mouse anti-nephrin monovalent antibody (mAb 5–1–6) (17, 31, 44). A total of 24 rats were injected, and six rats each were killed 1 h, 24 h, 5 days, and 20 days after injection. Twenty-four-hour urine samples were collected on 1, 3, 5, 7, 14, and 20 days after injection. Twenty-four-hour urine samples were collected on 1, 3, 5, 7, 14, and 20 days after injection. Urinary protein concentrations were measured by colorimetric assay with Protein Assay Reagent (Bio-Rad) using bovine serum albumin as a standard. In both models, small tissue samples were used for immunofluorescence studies. To prepare two sets of glomerular RNA in each time point, glomeruli were isolated from the remaining kidney tissue pooled from three rats each by a sieve method (21), and glomerular RNA was prepared with Trizol. To prepare another set of glomerular RNA, three rats were injected with PAN, and kidneys were removed at 24 h after the injection.

Immunohistochemistry. Immunohistochemical studies were performed basically according to the method previously reported (17). The 3-μm-thick sections with cryostat of frozen rat and human materials or cultured podocytes on glass coverslips were fixed with acetone for 1 min at room temperature and incubated at 37°C for 30 min and at 4°C overnight with rabbit anti-neurexin antibody (reaction to amino acids 1109–1123; WPANDPRSTRSDRLA) (Calbiochem, San Diego, CA) and stained with FITC-conjugated swine anti-rabbit IgG (DAKO, Glostrup, Denmark) at 37°C for 30 min. The anti-neurexin antibody recognized both neurexin-1α and -1β. The sections were observed with immunofluorescence microscopy (BX-50; Olympus, Tokyo). The specificity of anti-neurexin antibody was confirmed by preabsorption analysis with a peptide used for preparation of this antibody. The peptide for preabsorption analysis was purchased from GenScript (Scotch Plains, NJ). To evaluate the alterations of neurexin staining in the injured rat glomeruli, the staining was graded semiquantitatively as follows: continuous staining of >75% of the area of the capillary loops was graded 4; 50 to 75% was graded 3; 25 to 50% was graded 2; and 0 to 25% was graded 1. A score was assigned to each glomerulus, and more than 30 glomeruli of each rat were analyzed. The final score was shown as the mean. To analyze the localization of neurexin in glomeruli, the dual-labeling immunofluorescence study was performed basically according to the method of the previous report (10). The following primary antibodies were used to label the glomerular cell markers: 1) mouse anti-rat endothelial cell antigen-1 (RECA-1) antibody (Sero Tec, Oxford, UK); 2) mouse monoclonal antibodies to Thy 1.1, a mesangial cell marker (mAb 1–22–3) (15); and 2) mouse mAb against nephrin, a podocyte marker (mAb 5–1–6), goat antibody against CD2AP, a podocyte marker (Santa Cruz, CA). The secondary antibodies were used as follows: 1) tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates) for anti-RECA-1 and mAb 5–1–6; 2) TRITC-conjugated goat anti-mouse IgG3 (Southern Biotechnology Associates) for mAb 1–22–3; and 3) TRITC-conjugated donkey anti-goat IgG (Proteo Immunoresearch, San Francisco, CA) for anti-CD2AP.

Real-time RT-PCR. Real-time RT-PCR was performed as described previously (30). The reactions and runs were performed three times for each sample. To prepare standard materials, PCR products of neurexin-1α, neurexin-1β, and GAPDH were subcloned into the plasmid vector pCRII-TOPO, and the plasmids were diluted from 1×10^10 copies to 1×10^7 copies to generate calibration curves, which were based on the linear relationship between the crossing point cycle values and the logarithm of the starting copy number.
followed by immunoblotting with goat anti-CD2AP antibody and mouse anti-CASK/LIN-2 (Zymed Laboratory, San Francisco, CA). Alkaline phosphatase-conjugated rabbit anti-goat IgG and rabbit anti-mouse IgG (Science Bio) were used to detect CD2AP and CASK, respectively.

Analyses of splice variants expression. Neurexin-1α has five splice sites in its extracellular site [splice site 1: amino acid 258–277, splice site 2: 379–393, splice site 3: 790–799, splice site 4: 1247–1276, splice site 5: 1410–1411 of rat neurexin-1α (accession no. Q63373)], and it is reported that several splice variants of neurexin-1α were detected in some tissues. To analyze the expression of the splice variants at sites 1–5 of rat glomeruli and cerebrum, the specific primers for neurexin-1α were designed as follows: to detect splice site 1 (sense, 5′-GAG GAC CCG TTT CCGCAA AGA C-3′; antisense, 5′-CGC TTC AAA GGC CCC TGA TCC C-3′); splice site 2 (sense, 5′-GGG ATC AGG GGC CTT TGA AGC G-3′; antisense, 5′-CAT CTT CCG GTG TTG GCC ATC CTT C-3′); splice site 3 (sense, 5′-GGA ACA GAT ACG TCT GTG ATT GCT C-3′; antisense, 5′-GCC ACT GAT TGT CGT TGA GTG G-3′); splice sites 4 and 5 (sense, 5′-GCC ACT GAT TGT CGT TGA GTG G-3′; antisense, 5′-CAT CCG GTG TTG GCC ATC CTT C-3′). The size of the bands of the PCR products was analyzed. To analyze whether the PCR products contain the splice sites 2, 4, and 5, cDNA of the PCR products containing these sites were sequenced. The sequencing was performed basically in accordance with the method previously reported (18). The PCR products were cloned into a TOPO Vector (Invitrogen), and DNA sequences were determined by an automated DNA sequencer (ABI 310; Perkin-Elmer Japan, Urayasu, Japan).

RESULTS

Detection of mRNA expression of neurexin-1α and -1β in several organs and tissues. The mRNA expression for neurexin-1α and -1β was detected in several rat organs and tissues of normal adult rat. No neurexin-1α signals could be detected in the rat pancreas or spleen (Fig. 1). The mRNA expression of neurexin-1α in whole kidney material is more extensive than that in the glomeruli, whereas the expression of neurexin-1β in the whole kidney material is less than that in the glomeruli.

Neurexins are expressed in podocytes. Neurexin staining in the kidney was restricted to the glomeruli, and it was found to be a discontinuous linear pattern along the glomerular capillary loops. No staining was observed in normal rabbit serum and in anti-neurexin antibody preabsorbed with the immunizing peptide (Fig. 2A). To analyze the localization of neurexins in the glomeruli, a dual-labeling immunofluorescence study was carried out with the following glomerular-cell markers: 1) the endothelial-cell marker RECA-1, 2) the mesangial-cell marker Thy 1.1 (mAb 1–22-3), and 3) the podocyte slit diaphragm marker nephrin (mAb 5–1–6). Neurexins were not observed in endothelial cells or mesangial cells but were present in podocytes (Fig. 2B). The localization of neurexins coincided almost completely with that of CD2AP. The neurexin staining did not coincide with nephrin staining in normal adult rat glomeruli.

The mRNA expression of neurexin-1α and -1β was already detected in embryonic kidney samples on embryonic date 13.5 (E 13.5), although no positive staining was detected with immunohistochemical analysis. Neurexin staining was first detected in the presumptive podocytes of the capillary loop-stage glomeruli. The staining was detected at the basal surface of podocytes, and was colocalized with nephrin (Fig. 2C). The mRNA expression of neurexin-1α and -1β was detected in mouse-cultured podocytes.

Neurexin staining was observed along the glomerular capillary loops in human kidney sections in a similar pattern to that in the adult rat section (Fig. 3).

Neurexins in podocytes have an interaction with CD2AP and CASK. Specific bands for CD2AP and CASK were detected in the precipitated material of anti-neurexin antibody (Fig. 4).

Expression of a single form of the splice variants of neurexin-1α in rat glomeruli. The PCR products of neurexin-1α containing the splice sites 1–5 from glomeruli and cerebrum are shown in Fig. 5A. In the analysis of the splice site 2, a single band of a PCR product without an insert was detected in the glomerular RNA, and two bands of the PCR products with and without the insert were detected in the cerebrum RNA. Amino acid sequences of these PCR products are shown in Fig. 5B. The PCR products of the glomeruli and cerebrum from the lower band completely lacked the splice site 2. In the analyses of the splice sites 1 and 3, a single band of insert plus was detected in the PCR products from both the glomerular and the cerebrum RNA materials. Two bands of the PCR products with and without an insert at the splice site 4 were detected in the mRNA from the cerebrum, but only a single band of the PCR product with the insert was detected in the glomerular RNA. It was confirmed by sequencing analysis that amino acids at the splice sites 4 and 5 were contained in the PCR product from glomerular RNA (Fig. 5B). The cDNA sequences of the intracellular site of neurexin-1α of the glomerular sample were completely identical to those of the cerebrum sample.

Expression of neurexins is altered in injured podocytes. The kinetics of proteinuria in PAN nephropathy and ANA nephropathy are shown in Fig. 6D. The representative immunofluorescence findings of neurexin in PAN and ANA nephropathy are shown in Fig. 6A. In PAN nephropathy, a decrease in neurexin staining was already detected at 24 h. The staining intensity was clearly reduced, and the staining pattern changed to a more discontinuous fine granular pattern on day 10, when proteinuria peaked. On day 28, when proteinuria was normalized, the staining intensity was not yet completely recovered. The staining pattern of neurexin on day 28 was linear at the basal surface...
of podocytes. The dual-labeling study showed that the neurexin staining on day 28 was colocalized with nephrin (Fig. 6C). In ANA nephropathy, the intensity of the neurexin staining decreased, and the staining pattern changed to become a more discontinuous pattern at 24 h. Almost no signal was detected in some glomeruli of rats on day 5, when the proteinuria peaked. Nephrin staining shifted to a discontinuous coarse granular pattern, and its staining intensity decreased on day 10 of PAN nephropathy and on day 5 of ANA nephropathy. The intensity of the nephrin staining was almost recovered on day 28 of PAN nephropathy and on day 20 of ANA nephropathy. No clear alteration was observed in the podocalyxin staining in PAN or ANA nephropathy. The results of our semiquantitative evaluation of the neurexin staining are shown in Fig. 6B.

The mRNA expression of neurexin-1α and -1β in PAN nephropathy was analyzed by real-time RT-PCR. The results were shown in Fig. 7. The mRNA expression of both neurexins was clearly decreased at 24 h after PAN injection. Neurexin-1α decreased to 5.0%, and neurexin-1β decreased to 21%.

DISCUSSION

Neurexins are type I membrane proteins that have been classified into two types, α and β. Neurexin-1α, the first neurexin identified, was isolated as a brain protein that bound to α-latrotoxin, a component of black widow spider venom. The other neurexins were discovered subsequently by virtue of their sequence similarity to neurexin-1α. Because it has been accepted that neurexins are neuronal cell-specific proteins (46), their expression in other tissues has not been precisely analyzed. In this study, we started to analyze the expression of neurexins in major organs. We found that neurexins were expressed not only in the neuronal tissues but also in several organs, including kidneys, lung, and colon (Fig. 1). We then analyzed the localization of neurexins in the kidney. Immunohistochemical studies demonstrated that neurexins are restrictedly expressed in the glomeruli in kidney. Neurexins in glomeruli were observed along the glomerular capillary loop as a podocyte pattern (Fig. 2). Dual-labeling studies with glomerular cell markers showed that neurexins were clearly apart from endothelial cell and mesangial cell markers. These observations showed that neurexins are expressed in podocytes. We observed with dual labeling immunofluorescence techniques that neurexins are colocalized with CD2AP, a component of the slit diaphragm (42) (Fig. 2). The immunoprecipitation assay with glomerular lysate demonstrated that neurexins have an interaction with CD2AP. We also confirmed that neurexins interact with CASK, a MAGUK family protein (Fig. 4). Neurexins have the four amino acid tail that functions as a recognition sequence for the PDZ domains of MAGUK. We confirmed that these four amino acids were conserved in the neurexin-1α cDNA synthesized from glomerular RNA. It is plausible that neurexins of the podocytes interact with
Both CD2AP and CASK are reported to interact with nephrin. These results suggest that neurexins are components of the slit diaphragm.

To elucidate the function of neurexin in the slit diaphragm, we analyzed the expression of neurexins in podocytes of the developing glomeruli and in the injured glomeruli. The mRNA expression of neurexins was already detected in the developing glomeruli on the embryonic date of 13.5, although their immunohistological expressions are not detected in the kidney tissue of this material. Because neurexins are not densely deposited in this stage, it is hard to detect the expression with immunohistochemical methods. The immunohistological expression of neurexins in the presumptive podocytes was first detected in the late capillary loop stage when the slit diaphragm structure is actively formed. The staining was detected at the basal surface of the podocytes that coincide with nephrin. For the analyses of the expression of neurexins in the injured podocytes, we adopted two proteinuric models: 1) PAN nephropathy, a mimic human minimal change nephrotic syndrome and 2) an ANA-induced nephropathy, which is a model of a pure slit diaphragm dysfunction. The intensity of the immunostaining of neurexins was clearly lowered in PAN nephropathy on day 10 when severe proteinuria was detected. An alteration in the expression of neurexins was also detected in

![Fig. 3. Expression of neurexin in the human kidney. Neurexin staining was detected in the human glomeruli. Staining was observed along the glomerular capillary loops. No staining was observed with NRS or with the anti-neurexin antibody preabsorbed with the immunizing peptide. Bar, 100 μm.](image1)

![Fig. 4. Findings of the interaction assay with the normal adult rat glomerular lysate solubilized with Triton-X-100. Specific bands of CD2AP and CASK were detected in the precipitated material with anti-neurexin antibody.](image2)

![Fig. 5. Expression of the splice variants of neurexin-1α. A: PCR products of the splice sites. The PCR products of the splice sites 1 and 3 were detected as a single band in both glomerular and cerebrum materials. Two bands of the PCR products of the splice site 2 were detected in the cerebrum material, and only an upper band was detected in the glomerular material. Two bands of the PCR products of the splice sites 4 and 5 were detected in the cerebrum material, but only a single band of the PCR product was detected in the glomerular material. B: amino acid sequence of the PCR products of sites 2, 4, and 5.](image3)
ANA-induced nephropathy. It should be noted that the alteration in neurexin expression is more rapid and clearer than that of nephrin staining even in the ANA-induced nephropathy. These observations show that neurexin can be an early sensitive marker to detect slit diaphragm injury. Neurexin is understood to be a presynaptic transmembrane protein and has a major role in cell-cell interactions across the synapse (47). The observations in the present study together with the reports of neurexin in the neuronal field suggest that neurexin is involved in the maintenance of the slit diaphragm, a podocyte-specific cell-cell interaction.

It is gradually becoming accepted that synaptic vesicle-like vesicles are expressed in a variety of cells and that these vesicles play a role in the molecular trafficking of cell-surface proteins. We and another group have reported that the synaptic vesicle-associated molecules were expressed in podocytes (8, 27, 35). It is reported that neurexins interact with synaptic vesicle-associated molecules (12, 34, 49). These observations...
suggest that neurexins in podocytes have an association with synaptic vesicle molecules and have a role in intracellular trafficking. In the present study, we showed that although the localization of neurexins is slightly apart from nephrin in normal adult glomeruli (Fig. 2B), the localization of neurexins coincided with that of nephrin in glomeruli on day 28 of PAN nephropathy when the slit diaphragm was under reconstruction (Fig. 6). We also observed that neurexin staining coincided with nephrin in the presumptive podocytes of the developing glomeruli (Fig. 2C). These observations suggest that neurexin plays a role in the intracellular trafficking of the slit diaphragm components.

The characteristic molecular nature of neurexins is that they exhibit a striking number of alternative splicing variants (26, 39, 43). Neurexins have three genes (neurexin-1, -2, and -3), each of which has two promoters, α and β. The constituents of neurexin-α comprise three overall repeats, a central epidermal growth factor-like sequence: 1) LNS (widespread sequence motifs in laminin A, neurexin, and sex hormone-binding globulin) domains; 2) an O-linked sugar attachment sequence (a single transmembrane region); and 3) a COOH-terminal cytoplasmic sequence. Neurexin-β has a short sequence and contains a single LNS domain, an O-linked sugar attachment sequence, a single transmembrane region, and a COOH-terminal cytoplasmic sequence (25). Neurexin-α has five splice sites (sites 1–5) and neurexin-β has two (sites 4 and 5) (46). Although it is reported that the splice variants have different ligands and play different roles, the localization and the role of each variant are not well understood. In this study, we found that only one form of neurexin-1α is expressed in glomeruli. Many forms of splice variants of neurexin-1α were detected in neuronal tissues, which suggests that the finding in glomeruli is unique. The form of neurexin with the motifs of the splice sites 1, 3, 4, and 5 and without the motif of the splice site 2 expressed in glomeruli may play a differential role in podocytes.

We confirmed that neurexin is expressed in the podocytes of the human kidney (Fig. 3). The identity of the amino acid sequence of neurexins between humans and rats is higher than 95% (45, 47). We believe that the findings on neurexins observed with rat materials are basically compatible with those of human neurexins. Further investigation of neurexins may provide a novel diagnostic method for glomerular diseases and may enable the establishment of a novel therapy for proteinuria.
In summary, the present study showed that neurexin is associated with the podocyte slit diaphragm, a key structure regulating the barrier function of glomerular capillary wall. We propose that neurexin can be an early diagnostic marker to detect podocyte injury.

GRANTS
This work was supported by Grant-Aids for Scientific Research (C) (18590886 and 20590950) to H. Kawachi) from Ministry of Education, Culture, Sports, Science and Technology of Japan.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES
6. Donoviel DB, Freed DD, Vogel H, Potter DG, Hawkins E, Barrish JP. Fractalkine expression and the recruitment of CX3CR1 by 10.220.33.6 on November 7, 2016 http://ajpregu.physiology.org/ Downloaded from


