Identification of a novel neuroligin in humans which binds to PSD-95 and has a widespread expression

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Neuroligins, first discovered in rat brain, form a family of three synaptically enriched membrane proteins. Using reverse transcription–PCR of human brain polyadenylated RNA and extensive database searches, we identified the human homologues of the three rat neuroligins and a cDNA encoding a fourth member, which we named neuroligin 4. Neuroligin 4 has 63–73 % amino acid identity with the other members of the human neuroligin family, and the same predicted domain structure. DNA database analyses, furthermore, indicated that a possible fifth neuroligin gene may be present in the human genome. Northern-blot analysis revealed expression of neuroligin 4 in heart, liver, skeletal muscle and pancreas, but barely at all in brain. Overexpression of neuroligin 4 cDNA in COS-7 cells led to the production of a 110 kDa protein. Immunofluorescence analysis demonstrated that the protein was integrated into the plasma membrane. Overexpression of cDNAs encoding neuroligin 4 and the PDZ-domain protein, PSD-95, in COS-7 cells resulted in the formation of detergent-resistant complexes. Neuroligin 4 did not bind to ZO-1, another PDZ-domain protein. Together, our data show that the human neuroligin family is composed of at least one additional member, and suggest that neuroligin 4 may also be produced outside the central nervous system.

Key words: cell adhesion, expressed sequence tag sequences, PDZ domain, synapse.

INTRODUCTION

The rat neuroligins consist of three integral plasma membrane proteins (neuroligins 1–3) with structural similarity in the extracellular portion to acetylcholinesterases, without having, however, catalytic activity [1,2]. Among each other, neuroligins have a related structural organization, with an N-terminal signal peptide, the esterase-like domain with two sites of alternative splicing (designated A and B), a small linker region of low sequence identity in front of the transmembrane domain, and a short cytosolic part with a highly conserved C-terminus. Biochemical isolation of neuroligin 1 from rat brain tissue shows co-purification with *N*-methyl-D-aspartate ('NMDA') receptor subunits and the postsynaptic scaffolding protein PSD-95. This suggests neuroligins to be enriched in synaptic regions, presumably in the postsynaptic membrane [1]. Using splicing variants of the synaptic plasma membrane protein, neurexin, as immobilized ligand, neuroligin 1 specifically binds to a particular splicing variant of β-, but not to α-neurexins [1]. Binding to β-neurexin was subsequently also shown for neuroligins 2 and 3 [2]. Overexpression of neuroligin 1 and β-neurexin in *Drosophila* S2 cells confers adhesive properties to these cells [3]. Via their intracellular C-terminus, rat neuroligins bind to the N-terminal region of PSD-95, the region encompassing the PDZ domains, but not to ZO-1, another scaffolding protein made of PDZ domains [4]. Immunogold electron microscopy analysis of rat brain sections has established a restriction of neuroligin 1 to the postsynapse [5], and double immunofluorescence analysis of neuroligin 1 and glutamate receptors has revealed a highly related distribution [5]. These findings suggest a predominant presence of neuroligin 1 at excitatory synapses, where the protein could form a heterophilic complex with β-neurexins [6].

Co-cultures of neuronal cultures and HEK-293 cells transiently overexpressing mouse neuroligins 1 or 2 have illustrated that neuroligins can play a critical role in inducing presynaptic differentiation [7]. Induction of differentiation is dependent on the presence of the acetylcholinesterase homology domain of neuroligin proteins. The inductive properties of neuroligins are further supported by the ability of recombinant β-neurexin to suppress presynaptic differentiation, both in the heterocellular system and in co-cultures between growing axons of pontine explants and their *in vivo* targets, cerebellar granule cell neurons [7], suggesting that neuroligin–neurexin interactions may be sufficient to induce formation of a synapse *in vivo*.

An estimate of the number of individual neuronal cell types has yielded a figure of approx. 500 [8]. Such an enormous variety would emphasize the existence of selective intercellular recognition systems able to distinguish among the different cell types and to specify the local position of newly formed synapses during neuronal development. Because of their sequence divergence and variety, neuroligins might present a family of molecules suitable for such a function. Based on this consideration, the aim of the present study was to investigate whether further neuroligins are present in humans. For this purpose we made extensive reverse-transcription–PCR (RT–PCR) and database analyses and discovered a novel neuroligin in human brain RNA which is able to bind to PSD-95. Additionally, sequences were identified suggesting the existence of a potential fifth neuroligin gene.

Abbreviations used: EST, expressed sequence tag; RT–PCR, reverse transcription–PCR.

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MATERIALS AND METHODS

Antibodies

Mouse monoclonal antibody against rat neuroligins 1 and 3 (clone 4F9) was purchased from Synaptic Systems (Göttingen, Germany). Mouse monoclonal antibody against human c-Myc (clone 9E10) was from Zymed Laboratories (Basel, Switzerland). Goat polyclonal antibody against rat PSD-95 was from Santa Cruz Biotechnology (Heidelberg, Germany). Secondary anti-mouse antibodies for immunocytochemistry (Cy3 conjugate) and for immunoblotting (peroxidase conjugate) were from Sigma.

RT-PCR

cDNA from human brain polyadenylated RNA (ClonTech Laboratories, Basel, Switzerland) was synthesized using the neuroligin 4/5-specific primer, R9hNL4 [5′-GGAAATAGGG-CAAAG**CTA**TACTC-3′, encompassing the stop codon (shown in **bold**)] and the Superscript Preamplification System kit from Life Technologies. A full-length product of 2.5 kb and a fragment of 658 bp were amplified on the cDNA by PCR using the neuroligin 4-specific primer, F11hNL4 [5′-ATGCAGATTTG-AACC**ATG**TCAC-3′, encompassing the start codon (shown in **bold**)] and R9hNL4, and the neuroligin 4/5-specific primer F5hNL4 (5′-ATTCCAGTATGTTTCAACAACCAC-3′) and R9hNL4 respectively. The reactions were performed in an MJ thermal cycler (PTC-100; BioConcept, Allschwil, Switzerland) using *Taq* DNA polymerase and a reaction buffer from Promega and an annealing temperature of 60 °C. The PCR products were purified from a 1% (w/v) agarose gel using the jetquick gel extraction spin kit from Genomed (Chemie Brunschwig, Basel, Switzerland), and the 658 bp fragment was directly sequenced using primer F5hNL4.

Northern blotting

Northern-hybridization analysis was performed using a human tissue polyadenylated RNA blot from ClonTech Laboratories. A 3 kb RNA probe of human neuroligin 4 (containing the last 0.5 kb of the coding sequence and the 2.5 kb of the 3′ non-translated region) and a 0.75 kb RNA probe of human β-actin were prepared using the DIG RNA labelling kit from Roche Diagnostics (Rotkreuz, Switzerland). The membrane was pre-hybridized at 68 °C for 2 h in DIG Easy Hyb solution (Roche Diagnostics) in a hybridization tube. The RNA probe was then diluted to 100 ng/ml in prewarmed hybridization solution, and the blot was incubated overnight at 68 °C. The membrane was washed twice for 5 min at room temperature in 2 × SSC/0.1% SDS, and twice for 15 min at 68 °C in 0.5 × SSC/0.1% SDS (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). Hybridized probe was then detected using the DIG chemi-luminescent detection kit from Roche Diagnostics. For stripping off the RNA probe, 0.5% SDS solution heated to 95 °C was poured over the blot and incubated for 10 min on a rocking platform.

Cell culture and transfection

The African green monkey kidney fibroblast-like cell line COS-7 (A.T.C.C. clone CRL-1651) was maintained in Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) fetal-calf serum, 2 mM L-glutamine and 40 μg/ml gentamycin, at 37 °C and under 5% CO_2 in humidified air. COS-7 cells were transiently transfected using calcium phosphate [9]. Briefly, 2×10^5 cells were seeded in 60 mm culture plates. After 24 h, the medium was replaced by fresh culture medium. DNA (4 μg) was mixed with 100 μl of 0.25 M $CaCl_2$, vortexed, mixed with 100 μl of 2 × Bes-buffered saline (50 mM Bes, 280 mM NaCl and 1.5 mM Na_2HPO_4, pH 6.95) and vortexed again. After a 15 min incubation at room temperature, the transfection samples were added to the plates (1.33 μg of DNA/ml of medium). The cells were incubated under 3.5% CO_2 for 16 h, then washed twice with PBS and incubated for another 24 h in culture medium at 5% CO_2 before further processing.

Electrophoresis and immunoblotting

Whole cell extracts were prepared by lysing the cells in extraction buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100 and protease inhibitors). After homogenization, protein concentrations in the extracts were measured by a Bradford assay (Bio-Rad Laboratories, Glattbrugg, Switzerland). Equal amounts of protein were mixed with SDS sample buffer [10] and boiled for 3 min before loading. Proteins were resolved by SDS/PAGE on a 8% (w/v) acrylamide gel and transferred to a nitrocellulose membrane (Schleicher and Schuell, Riehen, Switzerland) using standard methods. The quality of the transfer was controlled by Ponceau S staining of the membrane. All blots were incubated in Blotto solution [5% (w/v) non-fat dried milk and 0.1% Tween 20 in Tris-buffered saline (10 mM Tris/HCl, pH 8.0, 150 mM NaCl)] for 45 min at room temperature on a rocking platform. The membranes were then incubated for 90 min with the primary antibody diluted in Blotto solution. After washing three times for 5 min with Tris-buffered saline containing 0.1% Tween 20, the primary antibody was reacted with a peroxidase-conjugated secondary antibody in Blotto solution for 45 min. After washing three times for 5 min with Tris-buffered saline containing 0.1% Tween-20, and once with Tris-buffered saline, immunoreactive bands were detected by ECL® (Amersham Pharmacia Biotech), according to the manufacturer's instructions. For re-probing, the blots were incubated twice for 40 min at room temperature in stripping solution (200 mM glycine/HCl, pH 2.2, 0.1% SDS and 1% Tween 20). After washing three times for 5 min in Tris-buffered saline containing 0.1% Tween 20, and once in Tris-buffered saline, the filters were re-probed, beginning with the blocking step.

Deglycosylation

Cell extracts were prepared with 200 μl of ice-cold lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P40, 0.1% SDS and protease inhibitors) per 60 mm culture plate. After homogenization, the protein concentration was determined by the Bradford assay. Extracts (40 μg) were diluted with lysis buffer up to 50 μl, boiled for 10 min and put on ice. After the addition of 2 units of N-glycosidase F (Roche Diagnostics), the samples were incubated at 37 °C for 16 h. Analysis was carried out by immunoblotting.

Immunocytochemistry

Cells were seeded on to12 mm coverslips [coated with 1% (w/v) gelatin for 60 min] at a density of 2×10^4 in a 24-well plate. At 24 h after transfection, the cells were washed with PBS, fixed and permeabilized with acetone/methanol (1:1, v/v) for 10 min at

−20 °C, and air-dried at room temperature. The cells were then treated with blocking solution [2 % (w/v) non-fat dried milk in PBS] for 10 min at room temperature on a rocking platform, and washed with PBS. Incubation with the primary antibody in blocking solution was for 75 min. The cells were then washed three times for 5 min with PBS. The Cy3-conjugated secondary antibody was also diluted in blocking solution, and the cells were incubated for 60 min. After washing three times for 5 min with PBS, the coverslips were drained and mounted in Lisbeth's embedding medium [30 mM Tris/HCl, pH 9.5, 70 % (v/v) glycerol and 50 mg/ml *N*-propyl gallate). The coverslips were sealed with nail polish, and the preparations were examined by fluorescence microscopy with a Zeiss Axioplan 2 microscope.

Immunoprecipitation

Extracts were prepared with 200 μl of ice-cold extraction buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 % Triton X-100 and protease inhibitors) per 60 mm culture plate and incubated on ice for 20 min. After homogenization, the extracts were centrifuged at 10000 ***g*** and 4 °C for 10 min to remove debris, and the protein concentration in the supernatant was determined by the Bradford assay. To reduce the background caused by non-specific adsorption of irrelevant cellular proteins to Protein G–Sepharose, the cell lysates (500 μg of protein in 400 μl of extraction buffer) were incubated with Protein G–Sepharose (Sigma) at 4 °C for 45 min on a rotating wheel. After centrifugation at 12000 ***g*** for 2 min to remove the Sepharose, 5 μg of antibody was added to the supernatant, and the extracts were incubated at 4 °C for 60 min. The Protein G–Sepharose was then added, and the extracts were incubated at 4 °C for another 45 min. After centrifugation, the complexes were washed three times for 15 min with extraction buffer. The precipitation procedure was repeated twice using the same extracts. The complexes were then pooled and resuspended in 2 × SDS sample buffer, boiled for 4 min and put on ice. Sepharose was removed by centrifugation, and the samples were analysed by immunoblotting.

Electronic database information

Public sequences available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/) were screened using the following nucleotide sequence databases: non-redundant, unfinished high throughput genomic sequences, and human expressed sequence tags (ESTs), mouse ESTs and other ESTs.

RESULTS

Identification of human neuroligin 4 and sequence analysis

Based on the cDNA sequences encoding neuroligins 1–3 from rat, we performed database sequence searches on human ESTs and genomic sequences currently available to identify human neuroligins 1–3. Three entries (KIAA1070, KIAA1366 and KIAA1480; [11–13]) revealed more than 98 % amino acid (92 % nucleotide) sequence identity with the rat neuroligins 1–3, and thus are probably the human homologues. KIAA1070 encodes human neuroligin 1 with an insert in splice site B, but not in splice site A. Sequences encoding two possible inserts in splice site A were found in the chromosomal sequence AC069531 (insert A1) and by RT-PCR analysis (insert A2) respectively (Figure 1). KIAA1366 encodes an N-terminally truncated human neuroligin 2. Two EST sequences (AI337820 and AW205858) allowed us to unequivocally complete the sequence. Only one insert was found for splice site A (Figure 1). KIAA1480 is also a partial sequence; it is identical to cDNA AF217411 and gene AF217413, which were identified as human neuroligin 3 (Figure 1) [14]. A further entry, KIAA1260 [15], had significantly less, albeit still highest, identity with neuroligins, suggesting that it encodes a novel neuroligin. Based on this sequence, RT-PCR was performed with human brain polyadenylated RNA. Sequence analysis of the 658 bp PCR fragment revealed virtually a 100 % match to KIAA1260. Alignment of the protein sequence with the compiled protein sequences of the putative human neuroligins 1–3 demonstrated their close relationship and supported the idea that this PCR fragment and KIAA1260 encode a novel neuroligin, which we named neuroligin 4 (Figure 1).

Human neuroligin 4 is composed of an N-terminal signal peptide with a predicted length of approx. 43 amino acids [16], an esterase-like domain encompassing almost the entire extracellular part, a short linker just in front of the transmembrane domain, and a cytosolic region. As observed with the rat neuroligins 1–3 [2], highest sequence conservation was found in the esterase domain, whereas the linker to the transmembrane region is completely different among all known neuroligins (Figure 1). Pairwise neuroligin comparison within the human proteins indicated closest identity between neuroligins 3 and 4 (Table 1). This is also reflected by the similar lengths of the parts following the esterase domain (Figure 1).

The human neuroligin 3 gene consists of eight exons [14]. Exons 3 and 4 encode the alternatively spliced inserts in splice site A. Alternative sequences in splice site B were described for rat and mouse neuroligin 1 [1,7]. Examination of human genomic sequences covering region B showed that the corresponding nine amino acids were not encoded by an individual exon, but would arise from the usage of a second downstream splice donor site. In human neuroligins 2–4, such a second splice donor site was not found, and in human neuroligin 3 the donor site was immediately followed by an in-frame stop codon. Furthermore, within this region the usually high sequence identity among all neuroligins was not maintained. From these findings we suggest that splice site B does not exist in human neuroligin 1, and that the extra amino acids in rodents might arise from erroneous or rare usage of a downstream splice donor site, whereas in humans the upstream site might be non-effective.

Sequences encoding a putative human neuroligin 5

Within the sequence of the 658 bp PCR fragment, a second weak signal was found at 12 positions. In addition, in the course of our DNA database analyses, two classes of neuroligin 4-related

Table 1 Identity numbers of human neuroligin sequences

The amino acid sequences of human neuroligins 1–4 (NL1–4), excluding the inserts in splice site A, were aligned pairwise. The numbers shown represent the percentage identities.

	Identity (%)			
	NL1	NL2	NL3	NL4
NL1	100	62.8	68.9	71.2
NL2		100	63.9	62.6
NL3			100	73.4
NL4				100

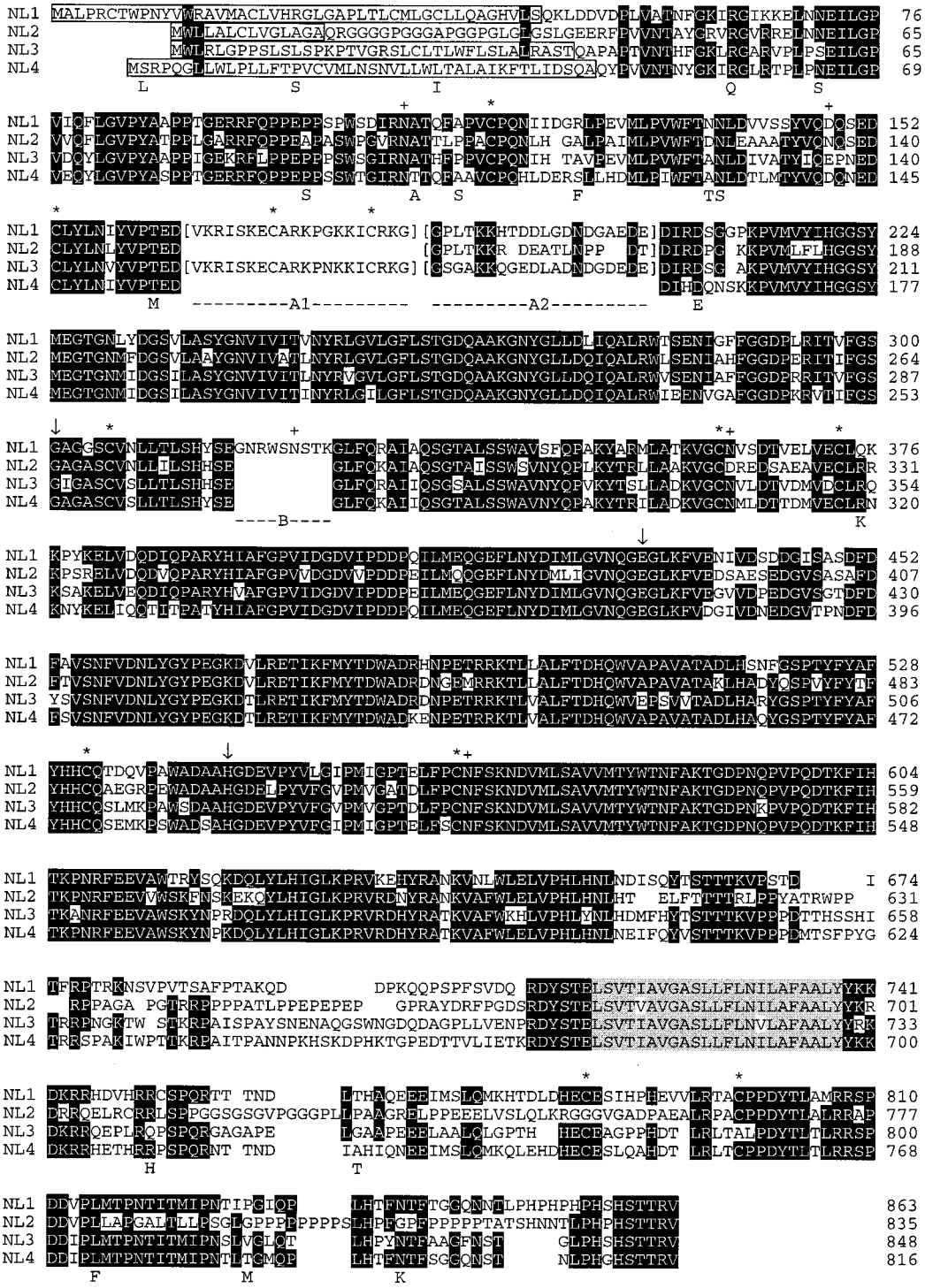

Figure 1 Sequence alignment of human neuroligins 1–4 (NL1–4)

Residues shared by at least three of the four human neuroligins are boxed on a black background. The predicted signal peptide sequences [16] are boxed on a white background (experimentally confirmed by sequencing only for rat neuroligin 2 [2]). The predicted transmembrane regions are boxed on a grey background. The Ser,Glu/Asp,His catalytic triad of esterases is marked by vertical arrows (in all neuroligins Ser is replaced by Gly). Conserved Cys residues are marked by asterisks. Positions of N-linked glycosylation consensus sequences (Asn-Xaa-Ser/Thr) are marked by +. Alternatively spliced inserts A1 and A2 are shown in brackets. Site B of neuroligin 1, which was found to be alternatively spliced only in rodents, is also marked. Amino acids differing between neuroligins 4 and 5 are indicated below the neuroligin 4 sequence. Numbers in the right-hand margin indicate amino acid numbers.

Table 2 Chromosomal assignment of human neuroligin genes

Chromosomal assignments were taken from the references indicated [done by radiation hybrid mapping (RHM)] or deduced from database analyses. The assignments of neuroligins 1–4 (NL1–4) are without contradictions. Inconsistent data exist for neuroligin 5 (NL5), as in [17] RHM resulted in chromosome 6, whereas database analyses using htgs determined the Y chromosome. Abbreviation: htgs, unfinished high throughput genomic sequences.

	Chromosome	RHM	Database analysis
NL1	3	[11]	htgs
NL2	17	–	[12]; htgs
NL3	X	[13]	[14]; htgs
NL4	X	[15]	htgs
NL5	6 or Y	[17] (6)	htgs (Y)

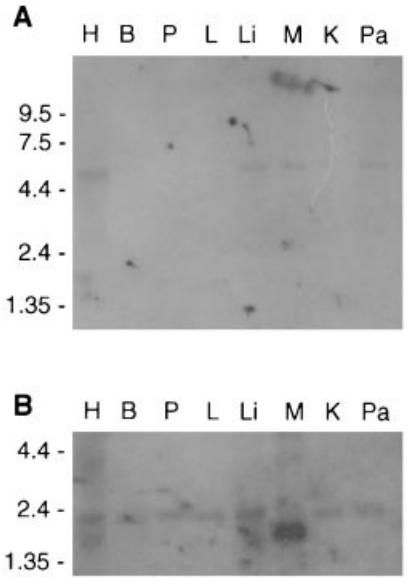

Figure 2 Northern-blot analysis of human neuroligin 4

(**A**) A human tissue polyadenylated RNA blot was hybridized with a 3 kb RNA probe of human neuroligin 4. Signals around 5.5 kb were detected in heart (H), liver (Li), skeletal muscle (M), pancreas (Pa) and eventually brain (B), but not in placenta (P), lung (L) and kidney (K). Size markers in kb are given at the left margin. (**B**) β-Actin expression in human polyadenylated RNA. The blot used in (**A**) was stripped and re-probed with a human β-actin RNA probe. Size markers are as in (**A**).

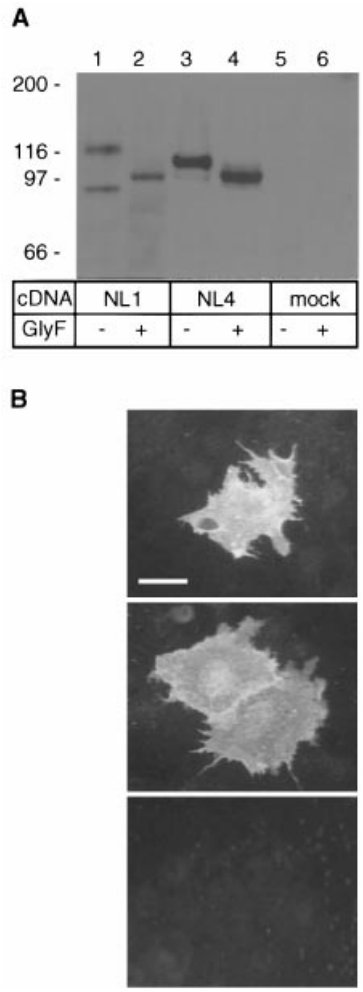

Figure 3 Overexpression of neuroligin 4 in transfected COS-7 cells

(**A**) COS-7 cells were transfected with vectors encoding neuroligin 1 (NL1; lane 1), neuroligin 4 (NL4; lane 3) or having no insert (mock, lane 5). Before SDS/PAGE analysis, 40 μg samples of the extracts were incubated either with (lanes 2, 4 and 6) or without N-glycosidase F (GlyF). Molecular-mass markers in kDa are shown on the left. (**B**) Immunofluorescence detection of neuroligin 1 (top panel) and neuroligin 4 (middle panel) in transiently transfected COS-7 cells. Bottom panel: mock-transfected cells. Scale bar is 10 μm.

sequences were discovered: class 1, with usually 100% identity to human neuroligin 4; and class 2, with, at most, 95% identity. Clones of the latter class, however, consistently revealed 100% identity to KIAA0951 [17] and to those 12 positions within the 658 bp PCR fragment. Further analysis of these sequences allowed the compilation of a complete open reading frame (N-terminally truncated clone KIAA0951 completed with chromosomal sequence AC010979), which could code for a fifth human neuroligin with approx. 97% identity to neuroligin 4. This neuroligin would differ from neuroligin 4 by only 19 amino acids (Figure 1). Most of these residues were clustered in the region in front of splice site A and at the C-terminus. Greater divergence was, however, observed when comparing the length and identity of available intron sequences of genomic neuroligin sequences. Between corresponding introns, sequence identity dropped to approx. 70–80%. Further evidence for two distinct genes came from the chromosomal assignment of human neuroligin genes (Table 2).

Expression of neuroligin 4 mRNA in human tissue

Neuroligins from rat were previously shown [1,2] to be predominantly, if not exclusively, expressed in brain tissue. To investigate the expression pattern of human neuroligin 4, we used a human tissue polyadenylated RNA blot and hybridized it with a 3 kb neuroligin 4 RNA probe encompassing most of the 3′-non-translated region, since this part had the lowest identity with the other neuroligins. Neuroligin 4 mRNA, with an approximate size of 5.5 kb, was found with the highest relative expression in heart. Lower expression levels were detected in liver, skeletal muscle and pancreas, whereas in brain, placenta,

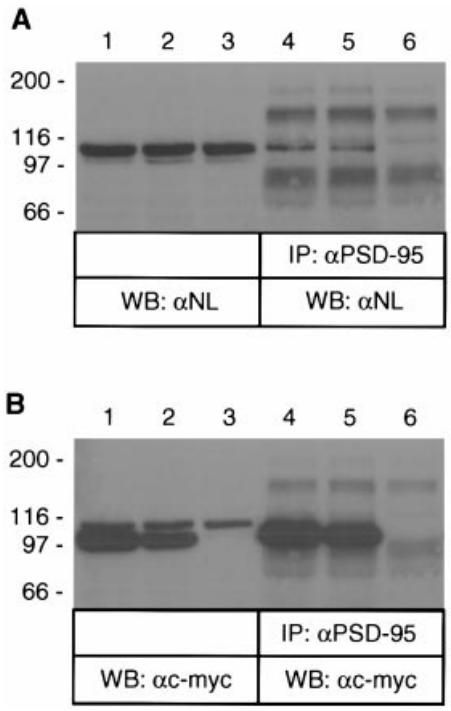

Figure 4 Association of neuroligin 4 and PSD-95 in transfected COS-7 cells

(**A**) Extracts in lanes 4–6 were immunoprecipitated with anti-PSD-95 antibodies (αPSD-95). Neuroligin (NL) 4 was detected in all lanes by immunoblotting with the anti-neuroligin antibody. Lane 1, 40 μg of protein from neuroligin 4-transfected cells mixed with Myc-PSD-95-transfected cells; lane 2, 40 μg of protein from double transfected cells; lane 3, 40 μg of protein from neuroligin 4-transfected cells mixed with mock-transfected cells; lane 4, immunoprecipitation of PSD-95 after mixing extracts of cells independently transfected with neuroligin 4 and Myc-PSD-95 cDNAs respectively; lane 5, immunoprecipitation of PSD-95 from double transfections; lane 6, control precipitation of extracts from neuroligin 4-transfected cells mixed with mock-transfected cells. Size markers in kDa are given at the left margin. (**B**) The blot used in (**A**) was stripped and re-probed with the anti-c-myc antibody to identify Myc-tagged PSD-95. The bands above PSD-95 in lanes 1–3 are residual signals of neuroligin 4. WB, Western blot; IP, immunoprecipitation.

lung and kidney, neuroligin 4 mRNA was hardly detectable, if at all (Figure 2A). The same blot was stripped and re-probed with a human β-actin RNA probe. Signals of similar intensities were detected in all lanes (Figure 2B).

Overexpression of neuroligin 4 in COS-7 cells

To investigate whether the neuroligin 4 cDNA encodes a mature protein, COS-7 cells were transiently transfected with cDNAs encoding either human neuroligin 1 or 4. At 24 h after transfection protein extracts were prepared and used for immunoblot analysis. To detect neuroligin proteins in COS-7 cells, we used the monoclonal antibody 4F9 [5], which reacts with the extracellular domain of rat neuroligins 1 and 3. No reactivity of the antibody was found with extracts from mock-transfected COS-7 cells (Figure 3A, lane 5). Neuroligin 1 was produced as a protein with an apparent mass of approx. 118 kDa in COS-7 cells (Figure 3A, lane 1). In cell extracts from neuroligin 4-transfected cells, the antibody reacted with a band of approx. 110 kDa, which is likely to be the respective neuroligin 4 protein (Figure 3A, lane 3). Treatment of these extracts with N-glycosidase F before electrophoresis reduced the apparent mass of both proteins to approx. 102 kDa (Figure 3A, lanes 2 and 4). The mass-shift of approx. 8 kDa after N-glycosidase F treatment is compatible with the presence of two consensus sequences for N-linked glycosylation in the neuroligin 4 sequence. Remarkably, neuroligin 1, with four consensus sites, shows the same phenomenon. It is presently unclear why the deglycosylated proteins have a higher apparent mass than calculated (91.9 kDa). Since endogenous neuroligin from mouse astrocytes showed the same phenomenon (M. F. Bolliger and S. M. Gloor, unpublished work), we suppose the mass difference to be caused by unusual migratory behaviour of the proteins, rather than by incomplete deglycosylation.

Immunofluorescence analysis of transiently transfected COS-7 cells resulted in delivery of the neuroligin 4 protein to the plasma membrane in a pattern indistinguishable from the distribution of neuroligin 1 (Figure 3B: top panel, neuroligin 1; middle panel, neuroligin 4; bottom panel, mock-transfected cells). Substantial quantities of the protein were also detected intracellularly in fixed and permeabilized COS-7 cells (Figure 3B). These data indicate that the neuroligin 4 cDNA encodes a protein which is glycosylated and integrated into the plasma membrane of COS-7 cells in the same way as neuroligin 1.

Association of neuroligin 4 with PSD-95

To further investigate basic properties of neuroligin 4, we tested its ability to interact with PSD-95, since rat neuroligins interact with this protein via their C-terminal ends [4] and all four human neuroligins contain the consensus motif -Thr/Ser-Xaa-Val required for binding to PDZ domains (see Figure 1) [18]. Separate COS-7 cell cultures were transfected with either neuroligin 4 or Myc-tagged PSD-95 cDNAs. At 24 h after transfection, detergent extracts for immunoprecipitation were prepared from each transfectant series, mixed, and immunoprecipitated with anti-PSD-95 or anti-c-Myc antibodies. Immunoblot analysis confirmed the presence of both proteins in the extracts (Figures 4A and 4B, lane 1). The co-precipitation of neuroligin 4 in the precipitate was probed by immunoblot analysis with neuroligin-specific antibodies. As shown in Figure 4(A, lane 4), neuroligin 4 was clearly detectable in the precipitate, providing evidence for its ability to interact with PSD-95. The same result was obtained when transfecting COS-7 cells with both cDNAs simultaneously (Figure 4A, lane 5), whereas no neuroligin immunoreactivity was discovered when omitting PSD-95 cDNA from the transfection (Figure 4A, lane 6). Re-probing the blot shown in Figure 4(A) with anti-c-Myc antibodies confirmed the presence of Myc-tagged PSD-95 in both extracts and precipitates (Figure 4B). Parallel experiments with endogenous ZO-1 instead of PSD-95 failed to co-precipitate neuroligin 4, suggesting a selectivity of the interaction between neuroligins and PDZ-domain proteins, as had been shown for rat neuroligins 1–3 [4].

DISCUSSION

In the present paper we present the identification of human neuroligin 4, a novel member of the neuroligin family of cell-surface proteins implicated in synapse formation. Several pieces of evidence support the identity of this protein. First, neuroligin 4 has highest sequence identity, in the order of 63–73 %, with the previously identified members of the family (Table 1), but only moderate similarity to other proteins, in particular esterases. Secondly, as in neuroligins 1–3, a serine residue essential for the

catalytic activity of esterases is absent in neuroligin 4 as well, and replaced by a glycine (Figure 1). The other two amino acids of the catalytic triad of esterases (serine, glutamic acid/aspartic acid, histidine), however, are preserved, as is the case for neuroligins 1–3. The fixed positions of all cysteine residues in neuroligins 1–3, some of which are thought to form disulphide bridges, are also completely maintained in neuroligin 4 (Figure 1). Finally, neuroligin 4, like the rat neuroligins 1–3 [4], binds to PSD-95, but not to ZO-1. Possible binding of neuroligin 4 (and 5) to neurexins remains to be investigated.

Northern-blot analysis demonstrated a tissue distribution of human neuroligin 4, which, unexpectedly, did not show a significant signal in total brain RNA, despite the fact that our cDNA and the clone KIAA1260 were derived from brain RNA. We suppose that this apparent discrepancy is most likely due to the different sensitivities of the methods used. Also, the broader expression profile of human neuroligin 4, in contrast with the nervous system-restricted expression pattern of rat neuroligins, may be due to different levels of sensitivity. It cannot be excluded, however, that expression of neuroligins may vary among different species. In support of this hypothesis are expression studies of human neuroligin 3, which report mRNA in heart, brain, skeletal muscle, placenta and pancreas [14,19], whereas, in rat tissues, signals were detected exclusively in brain [1,2]. Also of note in this context is the finding that the human neuroligin 3 probes detected a 7 kb transcript in the non-nervous system samples, but only a 4 kb transcript in brain [14,19]. In rat brain RNA, in contrast, comparable amounts of both the 4 kb and the 7 kb neuroligin 3 transcripts were found [2]. Additional evidence for a broader expression pattern of human neuroligins came from the observation that deposited human EST sequences were derived from many different tissues including heart, which revealed the strongest signal in our investigation.

The evidence in support of the expression of a fifth neuroligin is fourfold: (i) sequences encoding neuroligins 4 or 5 are derived from cDNAs; (ii) these sequences can clearly be assigned to either neuroligin 4 or 5, thus reducing the incidence of random sequencing artefacts; (iii) intron and non-translated sequences derived from neuroligin 4 and 5 genomic sequences, respectively, show a lower degree of identity; and (iv) although not without contradictions, most available data assign neuroligin 4 and 5 genomic sequences to different chromosomes (Table 2). Since we discovered neuroligin 5 sequences after RT-PCR with poly-adenylated RNA from a female individual, assignment of the neuroligin 5 gene to the Y chromosome may be excluded. Expression studies with KIAA0951 (neuroligin 5) and KIAA1260 (neuroligin 4) suggested distinct patterns [15]. Whether neuroligin 4 (and 5) are translated from endogenous mRNA can currently not be assessed because the neuroligin antibody recognizes at least neuroligins 1, 3 and 4.

If neuroligins have a broader tissue distribution than previously anticipated, this raises the question of subcellular structures and possible functions with which non-nervous system neuroligins may be related. An interesting association in this context may be with subcellular structures that are involved in cell–cell interactions and intercellular communications, such as tight, adherens and gap junctions. Although these structures, in contrast to synapses, are symmetrical with respect to molecular organization and information exchange, neuroligins might play a role there which could be analogous to the one assigned in the synapse, i.e. as inducer/positioner or stabilizer of such cellular contact sites. Supporting this notion of a role of neuroligins in junctional functionality are our observations that human umbilical vein endothelial cells and mouse choroid plexus epithelial cells express neuroligins (M. F. Bolliger and S. M. Gloor, unpublished work). Choroidal epithelial cells have highly developed tight and, presumably, adherens junctions in order to maintain the barrier between blood and cerebrospinal fluid. In this context it is important to mention that gliotactin, a glial protein in *Drosophila*, which may be a homologue of neuroligins, is involved in maintenance of the glia-made insect blood–nerve barrier [20]. Interestingly, we have observed that mouse astrocytes in culture are able to express neuroligins (M. F. Bolliger and S. M. Gloor, unpublished work). Supporting our observations are findings made by Gilbert et al. [21], who reported expression of neuroligins 2 and 3 in dorsal root ganglia and in cultured Schwann cells. Since Schwann cells form the myelin of peripheral nerves, it is tempting to speculate that neuroligins may play a role in myelin compaction and/or stabilization in junctional structures related to endothelial and epithelial junctions. It has been described that oligodendrocytes in the central nervous system form tight junctional structures [22], and that these junctions are lost in animals devoid of the gene encoding the tight junction protein oligodendrocyte-specific protein/claudin-11 [23]. Whether neuroligins are present in such structures remains to be tested. Additionally, putative extracellular binding partners for neuroligins outside the nervous system have to be identified, since neurexins are expressed significantly only in brain [24]. A homophilic interaction seems unlikely, since *Drosophila* S2 cells stably overexpressing neuroligin 1 do not form aggregates [3]. Further experiments will be required to elucidate functional properties of non-neuronal neuroligin proteins.

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