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Neuronal Pentraxin Receptor, a Novel Putative Integral Membrane Pentraxin That Interacts with Neuronal Pentraxin 1 and 2 and Taipoxin-associated Calcium-binding Protein 49*

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We have identified the first putative integral membrane pentraxin and named it neuronal pentraxin receptor (NPR). NPR is enriched by affinity chromatography on columns of a snake venom toxin, taipoxin, and columns of the taipoxin-binding proteins neuronal pentraxin 1 (NP1), neuronal pentraxin 2 (NP2), and taipoxin-associated calcium-binding protein 49 (TCBP49). The predominant form of NPR contains an putative NH_2-terminal transmembrane domain and all forms of NPR are glycosylated. NPR has 49 and 48% amino acid identity to NP1 and NP2, respectively, and NPR message is expressed in neuronal regions that express NP1 and NP2. We suggest that NPR, NP1, NP2, and TCBP49 are involved in a pathway responsible for the transport of taipoxin into synapses and that this may represent a novel neuronal uptake pathway involved in the clearance of synaptic debris.

We identified two taipoxin binding proteins for a presynaptic-acting snake venom neurotoxin, taipoxin, that blocks recycling of synaptic vesicles (1, 2). Affinity chromatography of solubilized rat brain membranes on columns of immobilized taipoxin enriches two major proteins: (i) neuronal pentraxin 1 (NP1),[1] a neuronally secreted protein with homology to serum pentraxins (2), and (ii) taipoxin-associated calcium-binding protein 49 (TCBP49), a reticular calcium-binding protein (3). NP1 has homology to previously identified pentraxins, such as serum amyloid P protein and C-reactive protein, which are elevated in the serum during acute phase response. Although the exact functions of these previously identified pentraxins are not known, they have been shown to bind, in a calcium-dependent manner, a wide variety of ligands and have been proposed to mediate the uptake of bacteria, toxins, and extracellular debris (4, 5). Homology to serum pentraxins, as well as the presence of a cleaved signal peptide and *N*-linked glycosylation sites, suggests that NP1 is secreted. The abundance of NP1 mRNA and rarity of NP1 protein suggest that NP1 protein has a rapid turnover. We have proposed that NP1 has a role in uptake at the synapse and that NP1 mediates the uptake of taipoxin into neurons. By low stringency screening, we identified an additional neuronal pentraxin (NP2) in human that has 54% amino acid identity with NP1 and is expressed in brain and multiple other tissues (6). Potential homologs of NP2 have been identified in guinea pig as a sperm acrosomal protein, apexin/p50 (7, 8), and in rat as a neural activity-regulated pentraxin, narp (9). The second taipoxin-binding protein, TCBP49, binds calcium via six EF-hand calcium binding motifs and is localized to the lumen of reticular membranes in neurons and glia (3). It contains the carboxyl-terminal sequence HDEL which has been shown to occasionally mediate endoplasmic reticulum retention in mammalian cells (10–12). We have suggested that NP1 binds to synaptic material and is taken up into a compartment containing TCBP49 (2, 3). We have also suggested that NP1 allows the internalization of taipoxin or a taipoxin·NP1 complex and that this uptake plays a role in the molecular mechanism of taipoxin toxicity.

To identify other proteins in such an uptake pathway, we have chromatographed rat brain proteins on columns of immobilized taipoxin, recombinant TCBP49, and glutathione *S*-transferase-NP1/NP2 fusion proteins. Here we report the identification, cloning, and cDNA sequence of a protein, neuronal pentraxin receptor (NPR), that binds tightly to taipoxin, TCBP49, NP1, and NP2 columns.

EXPERIMENTAL PROCEDURES

Materials—Taipoxin was purified from *Oxyuranus scutellatus* venom by gel exclusion chromatography as described (13). ^{32}P-Labeled nucleotides were obtained from NEN Life Science Products. Restriction and DNA modification enzymes were from New England Biolabs and Promega. Sequenase was from U. S. Biochemical Corp. Protein molecular weight standards were obtained from Bio-Rad and RNA and DNA standards from Life Technologies, Inc. Peroxidase-labeled secondary antibodies were from Cappel. All other chemicals and proteins were of reagent grade and used without further purification.

Bacterial Expression and Purification of TCBP49, GST-NP1, and GST-NP2—A piece of TCBP49 cDNA coding for the entire TCBP49 protein minus its NH_2-terminal signal sequence was amplified by PCR using a 5′ oligonucleotide (GGTGGCCATGGCCAGCAAGGCGGAGGAGCTG) that creates an *Nco*I site and initiator methionine codon at nucleotide 293 of the cDNA, just 3′ to the sequence coding for the signal peptide, and a 3′ oligonucleotide (CATAAATGTAGATCTGGTAGCAAACTACAAAAGGC) to create a *Bgl*II site 3′ to the stop codon. After restriction digest, the *Nco*I-*Bgl*II PCR fragment was subcloned between the *Nco*I and *Bam*HI sites of the bacterial expression vector pET 11d (Novagen, Inc., Madison, WI). Bacteria containing this vector were induced with isopropyl-β-D-thiogalactopyranoside (14), pelleted, resuspended in buffer (below), and lysed by French press. rTCBP49 was purified by taipoxin chromatography. Taipoxin was coupled to activated CH-Sepharose 4B (Pharmacia Biotech Inc.) according to the commercial protocol. The column was equilibrated in 20 mM Hepes, pH 7.4, 100 mM NaCl, and 1 mM $CaCl_2$, and rTCBP49-containing bacterial supernatant was applied to the column in the same buffer with protease inhibitors. Flow-through was collected and saved. The column was washed with

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[1] The abbreviations used are: NP1, neuronal pentraxin 1; NP2, neuronal pentraxin 2; TCBP49, taipoxin-associated calcium-binding protein 49; NPR, neuronal pentraxin receptor; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; kb, kilobase(s).

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equilibration buffer. rTCBP49 was eluted with the same buffer containing 10 mM EDTA and concentrated with a centriprep 30 (Amicon). This procedure yields rTCBP49 that is 95–98% pure as judged by Coomassie staining.

The piece of rat NP1 cDNA coding for the entire NP1 protein minus its NH_2-terminal signal sequence was amplified by PCR using a 5′ oligonucleotide (TCCTGGGAATTCGGGCCCAGGATTTCG) that creates an *Eco*RI site just 3′ to the region encoding the signal sequence and a 3′ oligonucleotide (AGAGAGAGGATGGGTCGACGCACAAGCAGGTG) that creates a *Sal*I site 3′ to the stop codon. After restriction digest, the *Eco*RI-*Sal*I PCR fragment was subcloned into the bacterial expression vector pGEX KG (15), positioning the cDNA downstream of and in frame with the glutathione *S*-transferase gene. To create the GST-NP2 expression construct, a similar portion of NP2 coding sequence was amplified by PCR using 5′ and 3′ oligonucleotides (GCCGCTGGATCCCAGGACAGCCCGGCGCCCGG and GCCCTGGAATTCCATGGCAACAGCCTGATCCC) to create *Bam*HI and *Eco*RI sites, respectively, for insertion into the expression vector pGEX KT (16). For protein expression, bacteria containing the pGEX KG-NP1 or pGEX KT-NP2 plasmid were induced with isopropyl-β-D-thiogalactopyranoside. The bacteria was pelleted and lysed by French press in 20 mM Hepes, pH 7.4, 10 mM EDTA containing 0.25 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and pepstatin. Insoluble material containing most of the GST-NP1 and GST-NP2 proteins was pelleted, washed in 20 mM Hepes, 10 mM EDTA, and 1% Triton X-100, and pelleted. The pellet was resuspended in 20 ml of 10 mM Hepes, pH 7.4, 5 mM EDTA, and 8 M urea containing protease inhibitors and incubated with rotation for [illegible] Insoluble material was removed by centrifugation, and the supernatant was dialyzed at 4 °C against 10 mM Hepes, 5 mM EDTA until the final urea concentration was below 5 mM. The dialyzed material was centrifuged. The supernatant contained the GST-NP1 or GST-NP2 proteins at 90–95% purity as judged by Coomassie staining.

Identification of Taipoxin, TCBP49, NP1, and NP2 Binding Proteins by Affinity Chromatography—Taipoxin, rTCBP49, GST-NP1, and GST-NP2 were coupled to activated CH-Sepharose (Pharmacia) at approximately 2 mg of protein/ml of column resin and final column volumes of 6–10 ml. Columns were equilibrated in 20 mM Hepes, pH 7.4, 100 mM NaCl, 0.2% Triton X-100, 1 mM $CaCl_2$. 15–20 rat brains were homogenized in 200 ml 0.32 M sucrose, 1 mM EGTA containing protease inhibitors. To enrich for integral membrane proteins or proteins in secretory or uptake pathways, a solubilized membrane preparation was used for chromatography. Membranes were pelleted by a 150,000 × *g*, 1-h centrifugation and then solubilized in 200 ml of 20 mM Hepes, 1% Triton X-100, 1 mM EGTA. Insoluble material was removed by a second 150,000 × *g* centrifugation, and the supernatant was adjusted to 100 mM NaCl and 1 mM free Ca^{2+} and then applied to the top of a column. Columns were washed with 10 column volumes of equilibration buffer and then eluted sequentially with 2 column volumes each of the same buffer containing 200 mM, 400 mM, 600 mM, 800 mM, and 1 M NaCl. To remove proteins bound tightly to the column in a calcium-dependent manner, a final elution with 3 column volumes of buffer containing 1 M NaCl and 10 mM EDTA was performed. Each eluate was concentrated in a Centriprep 30 (Amicon) and then run on SDS-PAGE gels. Gels were stained with Coomassie Blue or specific antibodies by Western blotting. Solubilized brain membranes were also chromatographed over a control column of uncoupled Sepharose CL-6B (Pharmacia) using an identical procedure.

Amino Acid Sequencing, cDNA Cloning, and Sequencing—The 55- and 65-kDa proteins were purified by SDS-PAGE using a model 491 Prep Cell (Bio-Rad). Purified protein was concentrated and then cleaved with CNBr. Cleaved peptides were separated by Tricine SDS-PAGE and transferred to a solid support. Individual peptide bands were cut out and subjected to automated amino acid sequencing. Degenerate oligonucleotides were designed from the peptide sequences and used to amplify a 332-base pair fragment of the desired cDNA from a rat brain cDNA library (Stratagene number 936501). The amplified fragment was then uniformly ^{32}P-labeled and used as a probe to screen the same brain cDNA library. Several positive clones contained the amplified fragment and contained sequences coding for other determined peptides. cDNAs were sequenced by the dideoxy nucleotide chain termination method. Sequence data were searched against GenBank™ and SWISS-PRO data bases.

RNA Blotting and in Situ Hybridization—Poly(A)$^+$-enriched RNA was electrophoresed, blotted onto nylon membranes, and hybridized with uniformly ^{32}P-labeled DNA probes (17). *In situ* hybridization was carried out essentially according to the procedure described by Wilkinson (18). cRNA probes (antisense and sense) were prepared from NPR cDNA (nucleotides 660–1660 of NPR cDNA) in pBluescript using T7 and T3 polymerase. Hybridized sections were washed in graded concentrations of SSC, treated with ribonuclease A, dehydrated, dried, and covered with photographic emulsion. Developed emulsions were dried and counter-stained with Hoescht nuclear stain.

Production of Antibodies—Two antibodies were raised to NPR. NPR1 (to residues 472–494 of NPR, carboxyl terminus of NPR) and NPR2 (to residues 31–57, post-transmembrane domain) were raised against synthetic peptides coupled to keyhole limpet hemocyanin as described (19). An antibody to rat NP2 was raised against a synthetic peptide (residues 410–430, carboxyl terminus of NP2). NP1 antibodies were described previously (2).

Construction of Recombinant NPR and Analysis of NPR Glycosylation—NPR coding sequence, excluding the NH_2-terminal transmembrane domain, was amplified with 5′ and 3′ oligonucleotides (TATATATCCATGGCCAGCCCCGCGCGCGCGCTGCCC and CAAGGGAGGGATCCTGAATGAGGTGGCCCCTCATGCCTTAGC) designed to create *Nco*I and *Bam*HI restriction sites, respectively. The 5′ oligonucleotide was designed to create a construct that initiates translation at amino acid 30 of the mature protein. The amplified fragment was subcloned into the *Nco*I and *Bam*HI sites of pET 11d. BL21(DE3) cells transformed with this vector were grown and induced with isopropyl-β-D-thiogalactopyranoside.

To test for *N*-linked glycosylation, aliquots of the GST-NP1 column EDTA eluate containing approximately 25 μg of the NP1, NP2, and NPR mixture were incubated at 37 °C overnight with or without 0.2 unit of endoglycosidase F/*N*-glycosidase F (Boehringer Mannheim). An aliquot of each reaction was run on SDS-PAGE gels, along with an untreated sample and an aliquot of pET-NPR bacterial supernatant. Gels were transferred to nitrocellulose and stained with NPR1 or NPR2 antibodies.

RESULTS

Chromatography on Taipoxin, rTCBP49, GST-NP1, and GST-NP2 Columns Reveals a Novel Protein of 55–65 kDa—Chromatography of solubilized brain membranes on taipoxin columns greatly enriches NP1 and TCBP49, which can be eluted by removing calcium with EDTA (2). To search for less abundant proteins that might bind to taipoxin columns, we repeated the chromatography of solubilized brain membranes on a taipoxin column in the presence of 1 mM calcium and eluted with a NaCl step gradient (200 mM, 400 mM, 600 mM, 800 mM, 1 M) in 20 mM Hepes, pH 7.4, 1 mM calcium chloride, 0.2% Triton X-100. After the 1 M NaCl elution, the column was eluted with 1 M NaCl containing 10 mM EDTA to disrupt interactions dependent on divalent cations. Starting membranes, column flow-through, and a similar proportion of each column fraction were run on SDS-PAGE gels. Gels were stained with Coomassie or transferred to nitrocellulose and incubated with antibodies to NP1, NP2, and TCBP49 (Fig. 1). TCBP49 elutes in the 200 and 400 mM NaCl steps, while NP1 elutes in the final 10 mM EDTA step, suggesting that TCBP49 and NP1 have different affinities for taipoxin. Calcium-dependent chromatography of recombinant TCBP49 on taipoxin columns also enriches rTCBP49, indicating that TCBP49 does bind to taipoxin in the absence of NP1 (data not shown). We had previously suggested that NP2 was not enriched on taipoxin columns (6), based on the lack of immunoreactivity with an antibody to apexin (7, 8), the probable guinea pig homolog of NP2. We reinvestigated the potential enrichment of NP2 on taipoxin columns with an antibody specific to the carboxyl terminus (residues 410–430) of rat NP2/narp (9). Like NP1, NP2 is greatly enriched on taipoxin columns, is not eluted off taipoxin columns with 1 M NaCl, and is only eluted with EDTA (Fig. 1). In addition to NP1 and NP2, this fraction also contains proteins that run as two diffuse bands at 55 and 65 kDa. Preliminary peptide analysis suggested that this was in fact a novel protein, which we have now named neuronal pentraxin receptor or NPR. In Fig. 1, these protein bands have been stained with antibody NPR1 described below. Similar chromatography on Sepharose (Fig. 1) or Tris-conjugated CH-Sepharose (2) fails to enrich NP1, NP2, NPR, or TCBP49.

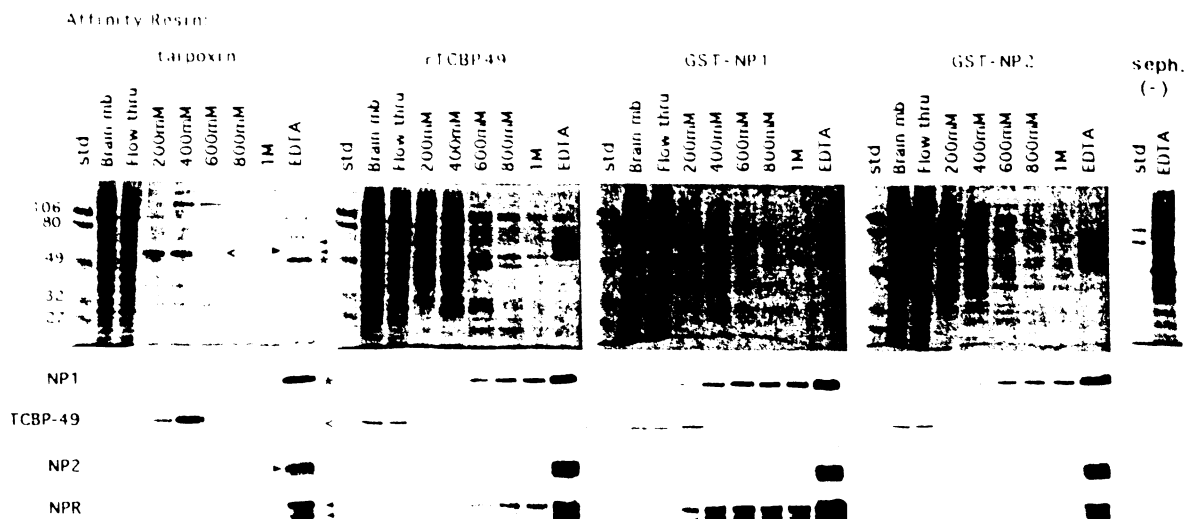

FIG. 1. **Taipoxin affinity chromatography: TCBP49, neuronal pentraxins 1 and 2, and NPR are purified on taipoxin affinity columns.** Chromatography of Triton X-100-solubilized rat brain membranes on columns of immobilized taipoxin, rTCBP49, GST-NP1, and GST-NP2 was as described under "Experimental Procedures." Solubilized brain membranes (20 μg), column flow-through (20 μg), and 1/10 of each column eluate (200 mM, 400 mM, 600 mM, 800 mM, 1 M NaCl, and 1 M NaCl containing 10 mM EDTA) were electrophoresed on 10% SDS-PAGE, and gels were stained with Coomassie or transferred to nitrocellulose membranes and immunoblotted with antibodies to TCBP49, NP1, NP2, and NPR. The positions of NP1, NP2, NPR, and TCBP49 are marked with an *asterisk* or *arrowheads*. Control chromatography on plain Sepharose (1/20 of 1 M NaCl, 10 mM EDTA eluate, 75 μg) is shown in the last panel. Apparent molecular mass of stained bands on these particular gels are: NP1, 49 kDa; TCBP49, 53 kDa; NP2, 60 kDa; and NPR, 55 and 65 kDa.

To investigate potential interactions between these taipoxin-binding proteins, we performed similar chromatographies on columns of immobilized TCBP49, NP1, and NP2. We expressed TCBP49, NP1, and NP2 in bacteria so that these proteins could be independently purified. Recombinant TCBP49 contains residues 23–318, deleting the signal peptide. We expressed NP1 and NP2 as glutathione *S*-transferase fusion proteins to aid in solubility. GST-NP1 and GST-NP2 proteins contain residues 19–432 of NP1 and residues 16–430 of NP2 fused at their amino termini to glutathione *S*-transferase. Purified rTCBP49, GST-NP1, and GST-NP2 were coupled to activated CH-Sepharose 4B according to commercial protocols. Solubilized rat brain membranes were chromatographed over these columns in the presence of 1 mM free calcium under the same conditions as chromatography over taipoxin columns. One major protein and several minor proteins in the 10 mM EDTA eluate are prominent by Coomassie Blue staining. The major protein is NP1 based on its size and immunoreactivity with three different NP1 antibodies (Fig. 1 and data not shown). This supports a direct calcium-dependent interaction between TCBP49 and NP1 in the absence of taipoxin. Chromatography on rTCBP49 also results in substantial enrichment of NP2 and multiple NPR bands, which elute with 10 mM EDTA. While we are looking at interactions facilitated by calcium, we cannot rule out that these interactions may also be mediated by other divalent cations.

Chromatography on GST-NP1 and GST-NP2 also results in the enrichment of NP1, NP2, and NPR in the 10 mM EDTA eluate (Fig. 1). TCBP49 is only slightly enriched by chromatography on GST-NP1 and GST-NP2 columns and elutes at 200 mM NaCl. Given the high enrichment of NP1 and NP2 on rTCBP49 columns, the lack of greater enrichment of TCBP49 on the GST-NP1 and GST-NP2 columns may reflect the lack of glycosylation of bacterially produced GST-NP1 and GST-NP2. Alternatively, the GST-fusion may interfere with TCBP49 interaction.

NPR Is an Additional, Putative Membrane-bound Member of the Neuronal Pentraxin Family—The enrichment of the 55- and 65-kDa protein(s) on taipoxin columns allowed purification of sufficient protein to obtain peptide sequence. Amino-terminal sequencing of the 65-kDa band yielded the sequence (MKFLA-VLLAAGMLAFLGAVI(C/S)(L/I)IASVPLA). This sequence contains a stretch of sufficient length (22–27 amino acids) and hydrophobicity to encode a transmembrane domain. The amino terminus of the 55-kDa band was blocked. The 55- and 65-kDa bands were purified and separated and were each treated with CNBr to obtain peptide fragments. CNBr digestion of the 55 and 65 kDa bands yielded similar peptides, suggesting the two bands represented variants of the same protein, although each digest contained a prominent unique peptide (data not shown). The unique peptide of the digest of the 65-kDa band gave sequence (LAFLGAVI(C/S)IIASVPLAASPARALP) that extended the sequence of 65-kDa band's amino terminus. The unique CNBr peptide from the digest of the 55 kDa band was blocked. Sequences of additional peptides common to the two digests were determined (DELEGQLLAKVLALEKERAALSH-GSHQQRQEVEKELDALQGRVAELEHG; ELLINDKVAQL-PLSLKDSNWHHI(C/S)IAWTTR). Amino acid sequence was used to design degenerate oligonucleotides which were used to amplify by PCR a 332-base pair fragment from a rat brain cDNA library. The PCR fragment was sequenced and determined to encode amino acid sequence between that encoded by the oligonucleotides used for PCR. The PCR fragment was used to probe a rat brain cDNA library to obtain several overlapping clones.

The longest cDNA obtained was approximately 5.5 kb in length and contained an open reading frame of about 1.5 kb (Fig. 2, GenBank™ number AF005099). As stated above, we named the protein(s) encoded by this cDNA neuronal pentraxin receptor or NPR. This cDNA does not contain an in frame stop codon in the 147-nucleotide 5′-untranslated region. The determined amino-terminal amino acid sequence of the 65-kDa protein matches the deduced amino acid sequence of the open reading frame except that the initial amino acid of the determined sequence is a methionine and in the deduced sequence is a leucine. This suggests that the CTG at nucleotide 148 is used as an initiator codon and codes for a methionine. The CTG at nucleotide 148 is present in all clones analyzed and is present in cDNA of mouse NPR, which has 96% amino acid identity with rat NPR (data not shown). The open reading frame contains all determined amino acid sequence from both the 55- and 65-kDa proteins. Additionally, an antibody (NPR1) to the car-

FIG. 2. **Nucleotide and translated amino acid sequences of message for rat NPR.** The sequences are numbered to the *right*, with the translated amino acid sequence being shown in single-letter code below the nucleotide sequence. The amino acid sequences obtained from protein fragments of a CNBr digest are *underlined*. The putative transmembrane domain is indicated by the *dashed underlined* and is contained within determined amino acid sequence. The deduced amino acid sequence is shown from the leucine at 1 by the CTG (which is *underlined*) at nucleotide 148. In the determined amino acid sequence of the 65-kDa NPR, this is a methionine. A putative downstream initiator ATG is also *underlined*. The translation stop codon is *underlined*. Potential sites for *N*-linked glycosylation are *boxed*.

boxyl terminus of this deduced amino acid sequence (residues 472–494) reacts with both the 55- and 65-kDa bands in the poxin column EDTA eluate (Fig. 1). 3′-Untranslated sequence of the cDNA clone is 3.9 kb in length. In this regard the message for NPR is similar to the message for NP1 (2). The deduced amino acid sequence is 494 residues long (Fig. 2). Hydropathy analysis reveals a single stretch of amino acids of sufficient length and hydrophobicity to encode a transmembrane domain. This stretch of 22–27 residues starts with the third residue of the protein. The deduced amino acid sequence of NPR contains three potential *N*-linked glycosylation sites at residues 42, 211, and 457.

We searched SWISS-PRO and GenBank™ data bases with the deduced amino acid sequence for NPR. The sequence of NPR has significant homology to the pentraxins and to neuronal pentraxin 1 and 2 in particular (Fig. 3). NPR has 49% amino acid sequence identity with rat NP1 and 48% identity with human NP2. As with NP1 and NP2, the carboxyl-terminal half of NPR has homology (22–25% identity) with the acute phase pentraxins C-reactive protein and serum amyloid P protein (20, 21). NPR's homology to neuronal pentraxins 1 and 2 is greatest in the carboxyl-terminal half of the protein (~63% identity) but is still significant in the amino-terminal half of the protein (~30% identity). The homology includes the putative calcium binding residues (22), the pentraxin signature sequence (HXCXS/TWXS (23)), and one glycosylation site. It has been suggested that larger pentraxins bind ligands via their more conserved carboxyl-terminal halves and that their amino-terminal halves confer unique functions upon these proteins (24). If this proves to be correct, NP1, NP2, and NPR may have similarities in ligand binding and distinct functions mediated by their amino-terminal domains.

NPR Message Is Brain-specific and Localized to Similar Brain Regions as NP1 and NP2—To determine the tissue distribution of NPR message, we probed Northern blots of rat tissue poly(A)$^+$ RNAs with a labeled random-primed NPR cDNA probe (Fig. 4). This NPR probe hybridizes to a single 5.5-kb mRNA. Overnight exposure of Northern blots reveals strong signal in brain and cerebellum lanes. This indicates that NPR mRNA is moderately abundant in neuronal tissues. One-week exposure of Northern blots reveals minimal or no signal in lanes of kidney, skeletal muscle, heart, liver, or testis poly(A)$^+$ RNAs, suggesting that NPR expression is highly brain specific. NPR cDNA probe also hybridizes to a 5.5-kb mRNA in nerve growth factor-induced and non-induced PC12 cells.

We investigated the cellular distribution of NPR message in the brain by *in situ* hybridization (Fig. 5). The highest hybridization is in the cerebellum and hippocampus. Like NP1, hybridization to NPR message is abundant in the Purkinje and granule neurons of the cerebellum and in CA3 neurons of the

FIG. 3. **Alignment of the amino acid sequence of NPR with NP1 and NP2.** The sequences are numbered on the *right*. Identical residues are *boxed*. Putative calcium binding residues are *shaded*. The pentraxin signature sequence is marked with an *overlying bar*. *RNPR*, rat neuronal pentraxin receptor; *RNP1*, rat neuronal pentraxin 1 (2); *HNP2*, human neuronal pentraxin 2 (6).

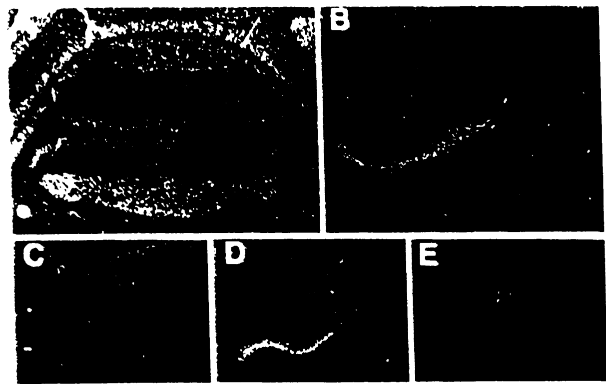

FIG. 5. **Distribution of NPR message in brain sections revealed by *in situ* hybridization.** Representative 7-μm sections of rat brain were hybridized to ^{35}S-UTP labeled NPR-riboprobes as outlined under "Experimental Procedures." Hybridization (detection of silver grains in the emulsion) is shown for a dark field image. NPR message is in the cerebellum (*A*), hippocampus (*B*), cerebral cortex (*C*), cingulate cortex (*D*), and taenia tecta (*E*). In the cerebellum hybridization is seen in Purkinje and granule neurons. In the hippocampus there is strong hybridization in CA3, moderate in the CA1, and low in the dentate gyrus (*DG*). In the cortex, hybridization is strongest in layer 6 of the neocortex and layer 2 of the olfactory cortex. Nuclei staining is not shown.

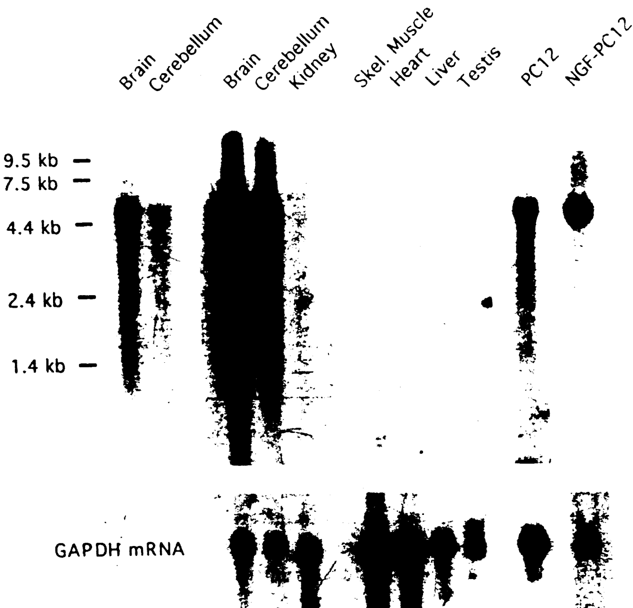

FIG. 4. **RNA blot analysis of message for NPR in rat tissues.** Poly(A)$^+$-enriched RNA (5 μg) from rat brain, cerebellum, kidney, skeletal muscle, heart, liver, and testis, as well as the rat neurosecretory cell line PC12 (uninduced and nerve growth factor-induced) were electrophoresed, blotted, and probed with a ^{32}P-labeled fragment of NPR cDNA (nucleotides 910–1241). The *top/left panel* is a 1-day autoradiographic exposure, the *top/middle panel* is 7 days, the two *top/right panels* are 13 days (uninduced) and 7 days (induced), respectively. The *bottom panels* shows hybridization of the same Northern to a cDNA probe to glyceraldehyde phosphate dehydrogenase (*GAPDH*).

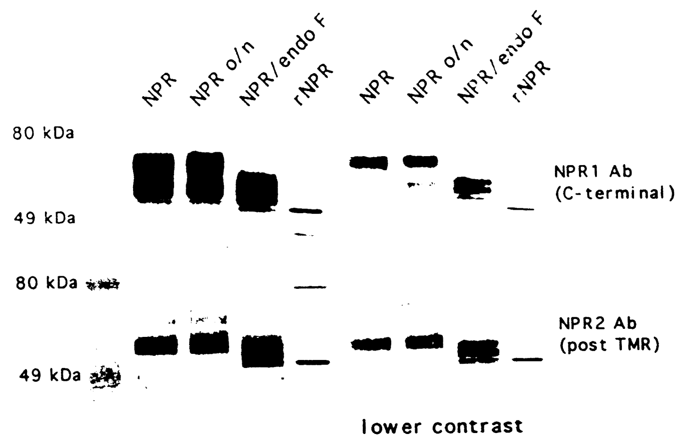

FIG. 6. **Deglycosylation of NPR by endoglycosidase F/glycopeptidase F.** Endoglycosidase F/glycopeptidase F treatment reduces the apparent molecular weight of all NPR immunoreactive proteins and only a small proportion is reduced to the size of recombinant NPR (residues 30–494). Aliquots of a column fraction containing NPR were incubated overnight at 37 °C in the absence (*NPR o/n*) or presence (*NPR/endo F*) of a mixture of endoglycosidase F glycopeptidase F. Native NPR or recombinant NPR was used as controls. Samples were run on SDS-PAGE gels, transferred to nitrocellulose, and blotted with either NPR1 (recognizes carboxyl terminus of NPR) or NPR2 (recognizes post-transmembrane region (*post TMR*) of NPR) antibodies.

hippocampus. It is present at moderate levels in dentate gyrus and CA1 neurons. Hybridization in the cerebral cortex is highest in layer 6 but is present diffusely in other layers. Hybridization to NPR probes is also present in the piriform cortex, taenia tecta, and cingulate cortex. There is a lack of hybridization in many midbrain regions, although at this level of detection we cannot rule out that these neurons may express low levels of NPR message. The pattern of hybridization to NPR message in the brain is similar to that of NP1 (2) and NP2 (9), although there are apparent differences in the pattern of hybridization between CA3 and CA1 regions of the hippocampus. Hybridization to NP1 probe is highest in CA3 and very low in CA1 (2). Hybridization to NPR probe is highest in CA3 but is still substantial in CA1. Reports of the hybridization of NP2 probe seem to suggest similar amounts in CA3 and CA1 (9).

NPR Is Glycosylated and Only a Small Proportion of Deglycosylated NPR Runs at the Apparent Molecular Weight of rNPR Lacking Its Transmembrane Domain—To investigate the differences between the 55- and 65-kDa NPR bands, we tested whether NPR is glycosylated as suggested by the presence of three consensus sites for *N*-linked glycosylation at residues 42, 211, and 457. We treated the EDTA eluate from a GST-NP1 column (containing NPR as described above) with endoglycosidase F and ran the treated eluate on SDS-PAGE gels. As a molecular size control we produced a recombinant protein con-

sisting of the entire NPR sequence after the apparent transmembrane domain (residues 30–494) and included this protein on the same gels. Proteins were transferred to nitrocellulose and stained with NPR antibodies (Fig. 6). Blots were incubated with an antibody NPR1 that recognizes the carboxyl terminus (residues 472–494 of NPR) or an antibody NPR2 that recognizes residues immediately after the transmembrane sequence (residues 32–[illegible] of NPR). Additionally, the peptide sequence recognized by NPR2 contains one of the three putative *N*-linked glycosylation sites of the protein (residue 42). 55- and 65-kDa NPR bands are recognized by both antibodies. However, while NPR1 appears to recognize 55- and 65-kDa bands equally well, NPR2 recognizes the 55-kDa protein well, but only very poorly recognizes the 65-kDa protein. This may suggest that the 65-kDa protein is glycosylated at residue 42, while the 55-kDa protein is not. Both antibodies strongly recognizes the 55-kDa protein, suggesting that this apparently smaller protein contains the full deduced amino acid sequence of NPR carboxyl-terminal to the proposed transmembrane domain. On incubation with endoglycosidase F, both bands shift to lower molecular weights, suggesting that NPR does contain *N*-linked glycosylation and consequently that all forms proceed through the secretory pathway. However, NPR immunoreactivity remains diffuse and appears to contain several distinct bands ranging from approximately 50 to 60 kDa in apparent molecular weight.

The multiple forms of NPR observed both before and after endoglycosidase F treatment do not appear to represent separate genes or alternative splicing, as we have identified only one type of cDNA and see only one message in Northern analysis. Additionally, the two major forms observed before endoglycosidase F treatment yield similar proteolytic products and apparently all forms are recognized by both carboxyl-terminal (NPR1) and post-transmembrane domain (NPR2) antibodies. As discussed, multiple forms of NPR are likely to be due in part to differential glycosylation; however, they may also represent proteolytic cleavage of the transmembrane domain or use of alternative initiation sites. In other proteins initiating with CTG, it has been found that this initiator codon is actually used as an alternative to a downstream ATG initiation site (25). Although no ATG codons are present in the 5′-untranslated region of the NPR cDNA, a downstream ATG at nucleotide 181 (methionine 12) could function as an initiation codon. NPR, if initiated at methionine 12, would have a hydrophobic domain of 13–19 residues that is bounded only carboxyl-terminally with charged residues. At present, additional experiments are necessary to determine if variability in the molecular weight of NPR is due to use of alternative initiation sites, proteolytic cleavage of the putative amino-terminal transmembrane domain, or other post-translational modifications.

DISCUSSION

We have previously identified two major taipoxin-binding proteins, NP1 and TCBP49 (2, 3), and suggested that they mediate the uptake and activation of taipoxin, a presynaptic-acting neurotoxin that blocks synaptic vesicle recycling (1, 2). We have shown that the addition of NP1 to glial cultures renders them susceptible to taipoxin toxicity (2), supporting a direct interaction between taipoxin and NP1 and a role for NP1 in the uptake of taipoxin. Additionally, the enrichment of rTCBP49 on taipoxin columns suggests that TCBP49 also interacts directly with taipoxin (data not shown). We now show that these two taipoxin-binding proteins are capable of binding to each other in the absence of taipoxin. Based on the homology of NP1 to acute phase pentraxins proposed to be involved in the uptake of pathogens and cellular debris, we have hypothesized that these proteins are components of a novel neuronal uptake pathway responsible for the clearance of synaptic debris and the uptake of taipoxin. Here we identify additional proteins that are likely constituents of this pathway. These include the previously characterized pentraxin, NP2, and a novel *putative* integral membrane pentraxin that we have named NPR.

Chromatography of solubilized brain membranes on columns of immobilized taipoxin greatly enriches NP1, TCBP49, and 2–3 minor proteins of apparent molecular masses between 50 and 65 kDa. One of these minor proteins is neuronal pentraxin 2 or NP2 (6). NP2 was identified from brain cDNA libraries by low stringency screening with NP1 probes and has also been identified as a guinea pig sperm acrosomal protein, apexin/p50 (7, 8), and as a neural activity regulated pentraxin, narp (9). With specific antiserum to rat NP2, we show that NP2 is also enriched on taipoxin columns. An additional minor protein enriched on taipoxin columns runs at both 55 and 65 kDa, and we now characterize it as NPR.

Northern analysis and *in situ* hybridization show that NPR is expressed in neurons. NPR is expressed in the same brain regions that express NP1 and NP2. All show the highest message expression in cerebellum and hippocampus and lower expression in the neocortex and pirriform cortex. The overlap of expression of message for NP1, NP2, and NPR in brain may reflect a functional interaction between these proteins. Message for the apparent rat homolog of NP2, narp, has been shown to be greatly up-regulated with high frequency stimulation of the hippocampus (9). Potential activity-dependent regulation of NPR or NP1 has not been investigated; however, NPR and NP1 normally have moderately abundant messages of 5–6 kb, while NP2 has a low abundance message of 2.5 kb.

NPR protein, like NP1 and NP2, is rare. Two different NPR antibodies fail to detect NPR in cortical and cerebellar homogenates or in brain membranes. The rarity of NPR protein contrasts with the abundance of NPR message. This discrepancy can be explained if NPR has a rapid turnover. Antibodies specific to the carboxyl terminus and post-transmembrane portion of NPR recognize both the 55- and 65-kDa NPR proteins. This demonstrates that all NPR proteins contain residues 30–494. The inability of antibody NPR2 (raised to a peptide containing a glycosylation site) to recognize the multiple forms of NPR equally well suggests that some of the NPR diversity is due to differential glycosylation. However, deglycosylation of NPR with endoglycosidase F yields multiple proteins of 50–60 kDa, suggesting that multiple NPR forms are not generated solely by differential glycosylation. Edman degradation of the 65-kDa NPR yielded clear sequence whereas Edman degradation of the 55-kDa NPR was blocked, suggesting that the 55- and 65-kDa NPR proteins have different amino termini. If NPR proteins differ in the first 30 residues, such differences are likely due to differential use of initiator codons or proteolytic cleavage. If initiated at methionine 12, NPR would have an amino-terminal hydrophobic domain of 13–19 residues. We currently cannot determine if the 55-kDa form of NPR contains residues 1–30, but endoglycosidase F cleavage produces only a small proportion of protein that runs at a similar molecular weight as recombinant NPR engineered to lack residues 1–30, its transmembrane domain. Expression of NPR in transfected CHO cells leads to cell surface exposure but not secretion,[2] suggesting that transfected NPR contains a transmembrane domain.

NPR exhibits several properties consistent with a role in uptake of NP1 and NP2. First, NPR, in at least the 65-kDa form, has an uncleaved amino-terminal sequence of sufficient length and hydrophobicity to be a transmembrane domain. This putative transmembrane domain and the presence of *N*-

[2] L. L. Kirkpatrick and M. S. Perin, manuscript in preparation.