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A rat brain cDNA encoding the neurotransmitter transporter with an unusual structure

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A rat cDNA clone encoding the novel membrane protein of the neurotransmitter transporters family was cloned and sequenced. The cDNA was identified as a transcript of the gene NTT4 of which a partial genomic clone was previously sequenced. Alignment of the amino acid sequence of NTT4 with other members of the neurotransmitter transporter family revealed a marked deviation from the conserved structure of all other members of the family. The largest extracellular loop with a potential glycosylation site was identified between membrane segments 7 and 8. The protein retains the common glycosylated loop between transmembrane helices 3 and 4 in all members of the family. The transcript of NTT4 was found exclusively in the central nervous system and is more abundant in the cerebellum and the cerebral cortex.

Neurotransmitter transporter; Central nervous system; Molecular cloning

1. INTRODUCTION

Cloning of cDNA encoding the transporter of γ-aminobutyric acid (GABA) [1,2] and subsequent cloning of a cDNA encoding the noradrenaline transporter [3] suggested the existence of a proliferated family of neurotransmitter transporters. Recently cDNAs encoding more than ten different neurotransmitter transporters have been cloned and sequenced [1–16]. The family of these genes could be divided into three subfamilies including the GABA and taurine transporters [12,15], the amino acid (glycine and proline) transporters [8–10], and the catecholamine transporters [3–7]. The general structure of all these gene products is very similar. They contain twelve potential transmembrane helices and an extended external loop with 3–4 glycosylation sites between membrane segments three and four [1–16]. The calculated molecular weights of the transporters is about 70 kDa and both their C- and N-terminal peripheral peptides contain about 40 amino acids and may be located on the cytoplasmic side of the membrane. In GABA and catecholamine transporter subfamilies the amino acid sequence of each member is 60–80% identical to the other members within a subfamily and about 40% identical to members between the two subfamilies [16]. Amino acid transporters such as the glycine transporter and proline transporter share about 40–45% homology with all members of the neurotransmitter transporter superfamily. Sequence homology among the members of the neurotransmitter transporters family gave clear indication that they evolved from a common ancestral gene. Moreover, partial genomic cloning of several neurotransmitter transporters revealed that in all of them the first intron in the reading frame is located in an identical position [16]. In this communication we report on the cloning and sequencing of cDNA of NTT4 which is a transcript of one of the reported genomic clones [16]. Extensive trials of expressing NTT4R cRNA in *Xenopus* oocytes failed to identify the specific substrate for the uptake. The structure of this gene product deviates from the common structure of the other transporters and a new mechanism of uptake may be involved by this transporter protein.

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2. EXPERIMENTAL PROCEDURES

2.1. *Screening of the rat brain cDNA library and sequencing*

A rat brain λZAP cDNA library (Stratagene) was screened at high stringency with the ^{32}P-labeled exon fragment of the genomic clone NTT4 [16] containing initiator methionine. The pBluescript plasmid was excised from the positive phages and analyzed by dot blot as previously described [15,17]. The cDNA was sequenced by the dideoxy chain termination method following serial deletions by exonuclease III [18,19]. Sequence verification was performed with synthetic oligonucleotide primers.

2.2. *Northern blot analysis*

Mouse brain was dissected into various parts and RNA was prepared from the various brain parts as well as from kidney and liver as previously described [15]. About 40 μg of total RNA were applied into an RNA denaturing agarose gel, and following electrophoresis and transfer onto a nylon filter was probed by the ^{32}P-labeled exon fragment of the genomic clone NTT4. The filters were washed and processed as previously described [15].

2.3. *Expression in* Xenopus *oocytes*

Six to 12 oocytes were assayed as previously described [15] for uptake of the following radioactive substances: adenosine, β-alanine, taurine, histamine, choline, γ-aminobutyric acid, glutamate, alanine,

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arginine, aspartate, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, cysteine, methionine, glutamine, tryptophan, proline, threonine and valine.

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   1 GAATTCGGCACGAGGCGCTGCGCTCCGCCCAGCTCCGTTCCGCTCCGCAGAGCCGACGCG   60
  61 GACCGGGCGCGGGGACAGCGGCACTCGGCTGGGGCCTTCGCAGCCACGCGCTCCCTCCGG  120
 121 GATCTGTACCCGGGCGGTCCCTAGAGCCCAGAGCGGGCAGAAGGCGGGAGTTGGGTTGGG  180
 181 ACTGGCGGCAACAGGGGAAGCGACGAGCCAGTTGATTCGCGTGCGCCGGCGCGCGCAGCT  240
 241 CCGGGTCCCGCAAGCCCCGGCTGGGGCCTGTTGCCGCTTGACTCCAGTTGCCCTCTGGAA  300
 301 GGACGGTGCACCCGCGACCCGTTGGGGACCACGGCCGCTCGGCGGGAATCCAGGGGCCGA  360
 361 GTCCGCGCGACGGCCCACGCGGGACAGGCTTTTGGATGAGAGAAAGCTGACAGGAGCTGA  420
 421 GCCCCACCTTCTCTGTGTGCTGGGGAATAGGGCTACGCAGCCCCAGTGGCACCCATGCCG  480
                                                     M  P
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     I  W  R  F  P  Y  L  C  Q  K  N  G  G  G  A  Y  L  V  P  Y
 781 CTGGTGCTGCTGATCATTATTGGCATCCCCCTCTTCTTTCTGGAGCTGGCTGTGGGCCAG  840
     L  V  L  L  I  I  I  G  I  P  L  F  F  L  E  L  A  V  G  Q
 841 AGGATCCGCCGTGGCAGCATTGGGGTCTGGCACTATGTGTGCCCCCGCCTGGGGGGCATT  900
     R  I  R  R  G  S  I  G  V  W  H  Y  V  C  P  R  L  G  G  I
 901 GGCTTTTCCAGCTGTATTGTCTGCCTCTTCGTTGGGCTGTACTACAACGTGATCATTGGA  960
     G  F  S  S  C  I  V  C  L  F  V  G  L  Y  Y  N  V  I  I  G
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     W  S  V  F  Y  F  F  K  S  F  Q  Y  P  L  P  W  S  E  C  P
1021 GTCATCAGGAATGGGACTGTGGCAGTGGTGGAGCCTGAGTGCGAGAAGAGCTCAGCTACT 1080
     V  I  R  N  G  T  V  A  V  V  E  P  E  C  E  K  S  S  A  T
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     T  Y  F  W  Y  R  E  A  L  D  I  S  N  S  I  S  E  S  G  G
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     T  Q  V  F  F  A  L  G  L  G  F  G  G  V  I  A  F  S  S  Y
1441 AACAAACAGGACAATAACTGCCACTTCGATGCTGCCTTGGTGTCCTTCATCAACTTCTTC 1500
     N  K  Q  D  N  N  C  H  F  D  A  A  L  V  S  F  I  N  F  F
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1561 AATGAGAAGTGTGTGGTCGAGAATGCTGAGAAAATTCTAGGGTACCTCAACTCCAATGTC 1620
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2101 GTGGCCTGGATTTACGGAACCAAGAAGTTTATGCAGGAGCTGACAGAGATGCTGGGCTTC 2160
     V  A  W  I  Y  G  T  K  K  F  M  Q  E  L  T  E  M  L  G  F
2161 CGACCGTACCGATTCTATTTCTACATGTGGAAGTTTGTGTCTCCACTGTGCATGGCTGTC 2220
     R  P  Y  R  F  Y  F  Y  M  W  K  F  V  S  P  L  C  M  A  V
2221 CTCACCACAGCCAGCATCATCCAGCTGGGGGTGTCACCCCCAGGCTACAGTGCCTGGATT 2280
     L  T  T  A  S  I  I  Q  L  G  V  S  P  P  G  Y  S  A  W  I
2281 AAGGAGGAGGCCGCCGAGCGCTACTTGTACTTCCCCAATTGGGCCATGGCACTGCTGATC 2340
     K  E  E  A  A  E  R  Y  L  Y  F  P  N  W  A  M  A  L  L  I
2341 ACCCTCATTGCTGTGGCAACCCTGCCTATCCCTGTGGTGTTCATCCTGAGGCACTTCCAC 2400
     T  L  I  A  V  A  T  L  P  I  P  V  V  F  I  L  R  H  F  H
2401 CTGCTCTCTGATGGTTCCAACACCCTCTCCGTGTCCTACAAGAAGGGCCGCATGATGAAG 2460
     L  L  S  D  G  S  N  T  L  S  V  S  Y  K  K  G  R  M  M  K
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     P  E  S  E  L  *
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2761 GATTGAGGATCTGTCCTGCCTTGTTCCCCACCCAAGTCCCGGCAGACTTCCTACCTATGA 2820
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2881 GAACTCTTGCCAAGTTGCAGCCATGTAACCACAACAATCCCAGTATGGGGGGACTTCAAG 2940
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3001 GTTTCAGGGAGGGTCCATTTGTGCCACTGGTCAATGTGGTGGTGGACAGAGGCTTCAGGG 3060
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3661 GTTTCTAAGGAAAGACAGGTAAACTCACTGCCCCCTTGGGCTGCCACTCACCTCGTGCCG 3720
3721 AATTC 3725
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Fig. 1. Nucleotide and deduced amino acid sequence of cDNA encoding NTT4R. The cDNA was cloned from the rat brain library and both strands were sequenced using the Exonuclease III digestion method [19] and oligonucleotide primers.

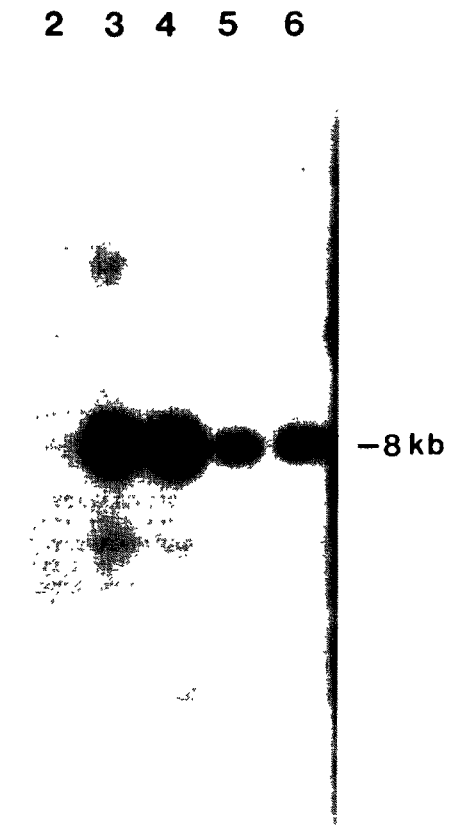

Fig. 2. Localization of NTT4 mRNA in various tissues. Total RNA was prepared from the various mouse brain parts and tissues as described in section 2. About 40 μg of RNA was applied in each well in a standard formaldehyde RNA gel [17]. Relative amounts of RNA in each sample were followed by the amounts of stained rRNA. The source of RNA was as follows: (1) liver, (2) kidney, (3) cerebellum, (4) cerebral cortex, (5) brainstem, (6) the rest of the brain.

3. RESULTS AND DISCUSSION

Sequence analysis revealed that four of the rat brain cDNA clones were highly homologous to the mouse NTT4 genomic clone [16]. We concluded that these cDNAs resulted from an identical gene of the rat genome and named the rate cDNA NTT4R. Fig. 1 shows the nucleotide and deduced amino acid sequence of the NTT4R clones. This sequence contains the entire reading frame but is missing parts of the 3′ and 5′ flanking regions. As shown in Fig. 2, the transcript size of NTT4 is about 8 kb. The size of the upstream flanking sequence of one of the NTT4R cDNA clones is estimated to be about 2 kb. This unusually long 5′ sequence may present difficulties for the expression of this

Fig. 3. Alignment of the amino acid sequences of NTT4R, the serotonin and taurine transporters. Deduced amino acid sequences of NTT4R (Fig. 1), the serotonin and taurine transporters [6,12] were aligned using a DNAstar program where NTT4R was aligned in two parts artificially separated in the middle of the loop between membrane segments 7 and 8 (see Fig. 4). Identical amino acids are indicated by (*).

cDNA. The *Eco*RI site starting the sequence depicted in Fig. 1 is present in the NTT4 transcript and does not result from a cloning artifact. Fig. 2 shows that the NTT4 RNA is quite abundant in the brain and is not present in the kidney or liver. The RNA is much more abundant in the cerebellum and cerebral cortex than in the brainstem or the rest of the brain. This suggests a neuronal function for NTT4. NTT4R encodes 727 amino acids which is about 100 residues larger than average size (about 600 amino acids) of other members of this family. The calculated molecular weight of the transporter (81 kDa) is greater than all known neurotransmitter transporters by about 10 kDa. The difference in molecular weight was demonstrated by comparison of in vitro translation of the cRNAs of NTT4R and the taurine transporter in rabbit reticulocyte lysate (not shown). The alignment of NTT4R, the serotonin and taurine transporters [6,12] is shown in Fig. 3. It is apparent that the extra sequences in NTT4 are present in the N- and C-termini and in the loop between membrane segments 7 and 8 (Fig. 4). The loop between membrane segments 3 and 4 contains 70– 90 amino acids in all transporters sequenced so far. It also contains 3–4 potential glycosylation sites [1–15]. In NTT4R this loop was shortened to about 60 amino acids and contains only a single glycosylation site. The loop between membrane segments 7 and 8 enlarged from 28 amino acids in the other transporters to 90 amino acids in NTT4R, and this loop of NTT4R exclusively contains a potential glycosylation site. Fig. 4 depicts a schematic proposal

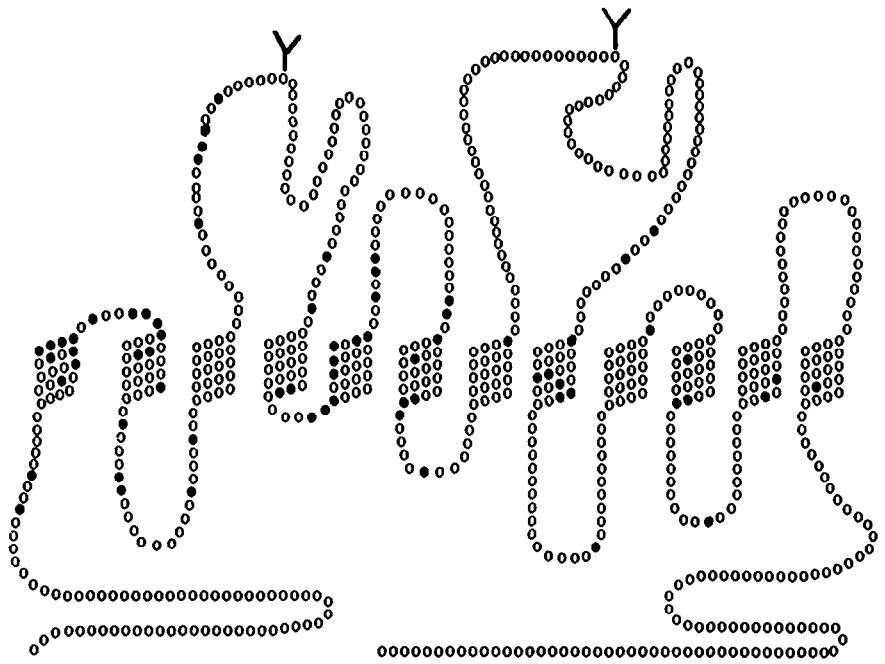

Fig. 4. Schematic presentation of the membrane structure of NTT4. Full symbols represent conserved amino acids that are present at the same position in all neurotransmitter transporters [1–15]. Y indicates a potential glycosylation site.

for the structure of NTT4. The full symbols indicate the position of amino acids that are conserved in all neurotransmitter transporters sequenced so far. NTT4R protein is the largest and least conserved member of the superfamily. The homology falls between 35–38% with other members of the family. The highest homology is with the choline transporter (37.9% identity) and the lowest homology is with the glycine transporters (35% identity). It has the sequence characteristics of amino acid transporters. The six transmembrane helices at the COOH-terminal half are less conserved for NTT4R than those at the amino-terminal half. Only 70 out of its 727 amino acids were strictly conserved among all the transporters. Their position is scattered all over the protein. The concentration of conserved amino acids in membrane segments 1 and 2 suggest a function common to all transporters. In addition, a notable conservation is present in the external loops between membrane segments 3 and 4 as well as segments 5 and 6. The protein folding may bring together these amino acid stretches, and together with the two conserved amino acids in the external loop near membrane segment 8, may form a vital recognition site on the external surface of the membrane.

An extensive attempt to identify the transported substances failed to reveal its identity. *Xenopus* oocytes injected with synthetic NTT4R mRNA show no uptake of individual and combination of the various amino acids, catecholamines and several other substances. The extended 5′ untranslated sequence may be a factor affecting the expression. NTT4R might transport an unknown neurotransmitter or require other facilitative ions besides Na^+, Cl^- and K^+. Alternatively, cofactors or hetero-oligomers may be involved in NTT4R functional expression. A more interesting possibility is that this protein is expressed in intracellular organelles depending on H^+ gradient and therefore evaded detection of its transport activity in *Xenopus* oocytes. Recently three laboratories reported on the cloning of an organellar transporter (SV2) [20–22]. Even though SV2 should be less specific than NTT4, no uptake could be detected in cells expressing this protein. The localization of NTT4 in the neuronal parts of the central nervous system and an unusual protein structure suggest an important role in neurotransmission.

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