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Isolation of cDNAs encoding a novel member of the neurotransmitter transporter gene family

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Degenerate oligonucleotides deduced from two regions of the rat γ-aminobutyric acid (GABA) transporter were used to amplify related sequences from rat spinal cord by polymerase chain reaction (PCR). The resulting product then allowed isolation of two overlapping cDNA clones with an open reading frame encoding a hydrophobic polypeptide of 630 amino acids. This protein, termed NTT, exhibits 38% sequence homology to the GABA and 48% to the noradrenaline transporters and contains 12 putative transmembrane regions. PCR amplification revealed low-level expression of NTT transcripts in spinal cord, but not in brain and cerebellum.

Neurotransmitter; Transporter; Polymerase chain reaction; Gene family; Spinal cord

1. INTRODUCTION

At most chemical synapses, neurotransmission is terminated by rapid re-uptake of the neurotransmitter from the synaptic cleft into the presynaptic terminal or surrounding glial cells. This translocation is mediated via transmitter-specific, sodium-coupled high-affinity transport systems [1]. The pharmacology and ion dependence of neurotransmitter transporters has been extensively studied by uptake assays with radiolabelled ligands using either plasma membrane vesicles and reconstituted systems [2–5] or *Xenopus* oocytes injected with poly(A)$^+$ RNA isolated from different regions of the central nervous system (CNS) [6,7]. These approaches have allowed the biochemical characterization and purification of the serotonin transporter [8], a partial enrichment of the glycine transporter [9], and purification of the GABA uptake system [4].

Amino acid sequences of isolated cyanogen bromide fragments from the purified GABA transporter have been successfully used to design oligonucleotides to clone and sequence cDNAs encoding the rat and the human GABA transporter (GAT1) polypeptides [10,11]. Using an expression cloning strategy, a cDNA clone encoding a human noradrenaline transporter (NET1) has also been isolated [12]. Overall identity between the amino acid sequences of both human transporters is 46%, and nearly identical Kyte-Doolittle hydrophobicity plots suggest 12–13 membrane-spanning segments of about 20 amino acids for these polypeptides. No significant similarity to Na^+/glucose and Na^+/proline carriers or to postsynaptic neurotransmitter receptors was observed [10,12]. These data indicate that the noradrenaline and the GABA transporters define a new membrane protein family. Here, we used the polymerase chain reaction (PCR) with oligonucleotides deduced from the rat GAT1 sequence to clone cDNAs from rat spinal cord which encode a novel member of the neurotransmitter transporter gene family.

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2. MATERIALS AND METHODS

2.1. *PCR amplification of related sequences*

PCR was performed on oligo(dT)-primed cDNA from rat spinal cord using the following degenerate oligonucleotides complementary to GAT1: 5′-AAGAACGG(T/G/C)GG(T/G/C)GG(T/G/C)GC(C/T)TTC-3′ (sense, corresponding to nucleotide positions 228–248); and 5′-CTG(G/A/T)GT(G/A)GC(G/A)GC(G/A)TCAAGCCA(G/C)AC-3′ (antisense, complementary to nucleotide positions 852–875). Reactions (100 μl) contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.01% (w/v) gelatine, ≈20 ng cDNA, 100 pmol of each primer, and 2.5 units of Ampli Taq Polymerase (Perkin Elmer Cetus). Amplification was for 30 cycles at 94°C for 30 s (denaturation), 50°C for 30 s (annealing) and 72°C for 2 min (extension). The resulting fragment (Eco640) was then processed as described in section 3.

2.2. *Isolation of cDNA clones*

A spinal cord cDNA library constructed in λZAP (7.5×10^5 pfu) was screened with the ^{32}P-labelled Eco640 fragment under the following hybridization conditions: 20% (v/v) formamide, 750 mM NaCl, 75 mM Tris-HCl, pH 7.5, 12.5 mM EDTA, 0.1% (w/v) SDS, 5× Denhard's solution and 100 μg/ml salmon sperm DNA at 50°C for 14 h. Filters were washed in 0.2×SSC, 0.1% (w/v) SDS at 65°C for 40 min. Bluescript cDNA was excised from the phages following the Stratagene manual and subjected to dideoxy sequencing [13] using synthetic primers.

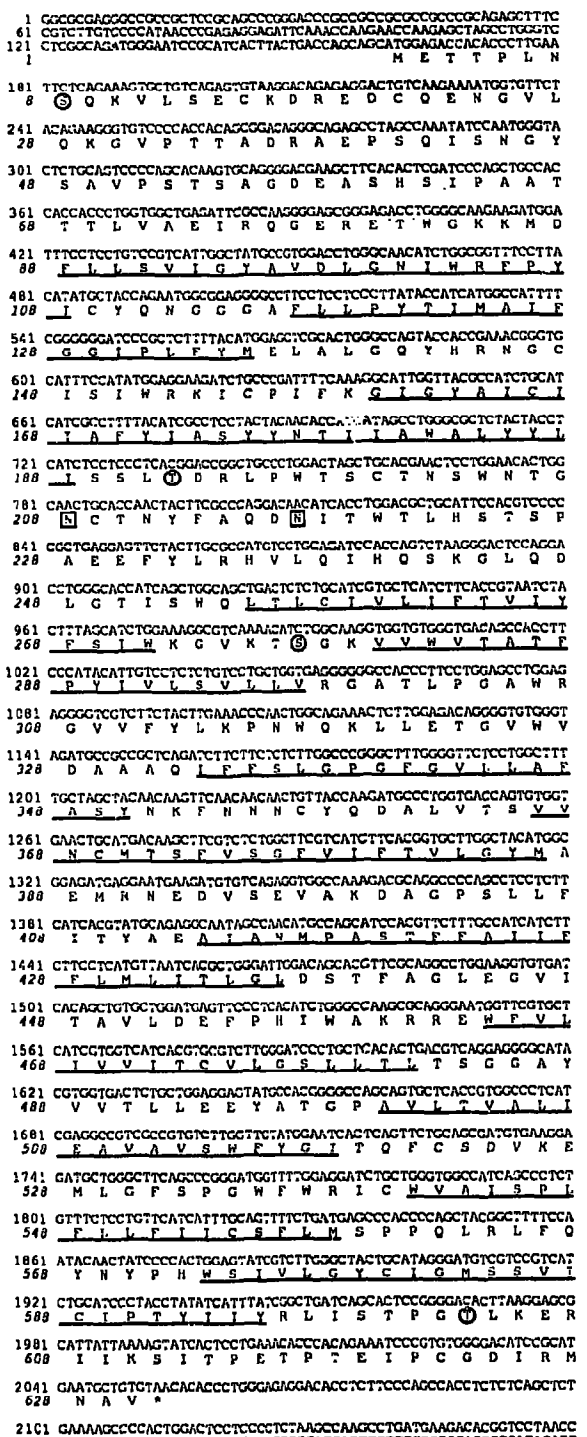

Fig. 1. Nucleotide and deduced amino acid sequences of the NTT cDNA. Predicted membrane spanning regions and two potential polyadenylation signals are underlined, *N*-linked glycosylation sites are boxed, and consensus sequences for phosphorylation by protein kinase C are circled. Numbering of nucleotides and of amino acids is indicated on the left side of the figure.

3. RESULTS AND DISCUSSION

Degenerate sense and antisense oligonucleotides covering selected sequences of the rat GAT1 cDNA (see section 2) were designed to encompass a region which contains a unique *Eco*RI restriction site at position 571. Using these oligonucleotides for PCR amplification of rat spinal cord cDNA we obtained a 640 bp fragment, which was subjected to *Eco*RI digestion. This resulted in two fragments of ≈300 bp and ≈340 bp corresponding to those expected from GAT1, and residual amplification product of 640 bp, which apparently did not contain the *Eco*RI restriction site. Analysis of the latter revealed a new cDNA sequence containing an open reading frame which exhibited remarkable amino acid homology to GAT1 and NET1. We then used this fragment, termed Eco640, to screen a λZAPI cDNA library prepared from rat spinal cord (kindly provided by Dr. Yves Maulet). Using high-stringency hybridization, two overlapping clones were isolated which covered the entire coding sequence for a novel member of the neurotransmitter transporter gene family, that hence was named NTT (neurotransmitter-transporter). As shown in Fig. 1, these clones specify an open reading frame of 1890 nucleotides, which encodes a protein of 630 amino acids with a calculated molecular weight of 70 171 Da. Potential polyadenylation signals are found at the 3′ end followed by a stretch of several A residues, and the nucleotides surrounding the presumptive start codon fulfill the criteria of eukaryotic translation start sites [14]. Within the deduced NTT protein, 2 possible *N*-linked glycosylation sites are located at amino acid positions 208 and 217, and 4 consensus phosphorylation sites for protein kinase C at positions 8, 192, 277 and 603.

Hydropathy analysis of the NTT polypeptide according to Kyte and Doolittle [15] resulted in a profile which is nearly identical to that of GAT1 (not shown). With 12 potential transmembrane regions (see Fig. 1), both the N- and C-termini may be placed into the cytoplasm, as no characteristic signal peptide is found in the N-terminal amino acid sequence.

Alignment of the deduced NTT protein sequence with the 2 known transporter polypeptides revealed significant homology along most of the sequence (Fig. 2). Calculated overall amino acid sequence identities of NTT to GAT1 are 38.2% and to NET1 47.7%, with a particularly high conservation in the putative membrane spanning segments.

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  1     METTPLNSQKVLSECKDREDCQENGVLQKGVPTTADRAEPSQISNGYSAVPSTSAGDEASHSIPAATTTLVAEIRQGERETWGKKMDFLLSVIGYAV  NTT
  1                                  MLLARMNPQVQPENNGADTGPEQPLRARKTAELLVVKERNGVQCLLAPRDGDAQPRETWGKKIDFLLSVVGFAV  NET1
  1                                                  MATDNSKVADGQISTEVSEAPVASDKPKTLVVKVQKKAGDLPDRDTWKGRFDFLMSCVGYAI  GAT1
                                                                                             * **   *** *  * *

 98 DLGNIWRFPYICYQNGGGAFLLPYTIMAIFGGIPLFYMELALGQYHRNGCISIWRKICPIFKGIGYAICIIAFYIASYYNTIIAWALYYLISSLTDRLPW  NTT
 75 DLANVWRFPYLCYKNGGGAFLIPYTLFLIIAGMPLFYMELALGQYNREGAATVW-KICPFFKGVGYAVILIALYVGFYYNVIIAWSLYYLFSSFTLNLPW  NET1
 63 GLGNVWRFPYLCGKNGGGAFLIPYFLTLIFAGVPLFLLECSLGQYTSIGGLGVW-KLAPMFKGVGLAAAVLSFWLNIYYIVIISWAIYYLYNSFTTTLPW  GAT1
     * * ***** *  ******* **     *  * ***  *  ****   *    * *  * *** * *            **  ** *  ***  * *  ***

198 TSCTNSWNTGNCTN------YFAQDNITWTLHSTSPAEEFYLRHVLQIHQSKGLQDLGTISWQLTLCIVLIFTVIYFSIWKGVKTSGKVVWVTATFPYIV  NTT
174 TDCGHTWNSPNCTDPKLLNGSVLGNHTKYSKYKFTPAAEFYERGVLHLHESSGIHDIGLPQWQLLLCLMVVVIVLYFSLWKGVKTSGKVVWITATLPYFV  NET1
162 KQCDNPWNTDRC-----FSNYSLVNTTNMT----SAVVEFWERNMHQMTD--GLDKPGQIRWPLAITLAIAWVLVYFCIWKGVGWTGKVVYFSATYPYIM  GAT1
      *   **   *                           ** *            *    *   * *          **  ****   ****   ** **

292 LSVLLVRGATLPGAWRGVVFYLKPNWQKLLETGVWVDAAAQIFFSLGPGFGVLLAFASYNKFNNNCYQDALVTSVVNCMTSFVSGFVIFTVLGYMAEMRN  NTT
274 LFVLLVHGVTLPGASNGINAYLHIDFYRLKEATVWIDAATQIFFSLGAGFGVLIAFASYNKFDNNCYRDALLTSSINCITSFVSGFAIFSILGYMAHEHK  NET1
251 LIILFFRGVTLPGAKEGILFYITPNFRKLSDSEVWLDAATQIFFSYGLGLGSLIALGSYNSFHNNVYRDSIIVCCINSCTSMFAGFVIFSIVGFMAHVTK  GAT1
    *  *   * *****  *    *      *     ** *** ***** * * * * *  *** * ** * *        *  **   ** **  * **

392 EDVSEVAKDAGPSLLFITYAEAIANMPASTFFAIIFFLMLITLGLDSTFAGLEGVITAVLDEFPHIWAKRREWFVLIVVITCVLGSLLTLTSGGAYVVTL  NTT
374 VNIEDVATE-GAGLVFILYPEAISTLSGSTFWAVVFFVMLLALGLDSSMGGMEAVITGLADDF-QVLKRHRKLFTFGVTFSTFLLALFCITKGGIYVLTL  NET1
351 RSIADVAA-SGPGLAFLAYPEAVTQLPISPLWAILFFSMLLMLGIDSQFCTVEGFITALVDEYPRLLRNRRELFIAAVCIVSYLIGLSNITQGGIYVFKL  GAT1
         **   *  *  *  * **    *  *  ** **  ** **   *  **  *       *  *  *     *  *   * ** **  *

492 LEEYAT-GPAVLTVALIEAVAVSWFYGITQFCSDVKEMLGFSPGWFWRICWVAISPLFLLFIICSFLMSPPQLRLFQYNYPHWSIVLGYCIGMSSVICIP  NTT
472 LDTFAA-GTSILFAVLMEAIGVSWFYGVDRFSNDIQQMMGFRPGLYWRLCWKFVSPAFLLFVVVVSIINFKPLTYDDYIFPPWANWVGWGIALSSMVLVP  NET1
450 FDYYSASGMSLLFLVFFECVSISWFYGVNRFYDNIQEMVGSRPCIWWKLCWSFFTPIIVAGVFLFSAVQMTPLTMGSYVFPKWGQGVGWLMALSSMVLIP  GAT1
            *   *    *   *****  *     * * *  *  **   *                    *    * *   *    **   *

591 TYIIYRLISTPGTLKERIIKSITPETPTEIPCG-DIRMNAV                                                           NTT
571 IYVIYKFLSTQGSLWERLAYGITPENEHHLVAQRDIRQFQLQHWLAI                                                     NET1
550 GYMAYMFLTLKGSLKQRLQVMIQPSEDIVRPENGPEQPQAGSSASKEAYI                                                  GAT1
     *  *    * * *  * *
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Fig. 2. Comparison of NTT, GAT1 and NET1 protein sequences. Amino acids identical in all transporters are marked by asterisks. Gaps were introduced to optimize identical sequence positions.

Northern analysis of poly(A)$^+$ RNA isolated from rat brain, cerebellum, and spinal cord failed to reveal detectable amounts of NTT transcripts in any of these tissues even after very long exposure times (>14 days; data not shown). PCR amplification of different rat cDNAs with NTT-specific primers encompassing nucleotide positions 154–178 (sense) and 672–693 (antisense) were therefore used for NTT mRNA detection. As shown in Fig. 3, NTT amplification product was only obtained with spinal cord cDNA, whereas no signals were seen with total brain or cerebellum. Control amplifications with β-actin primers produced equal signals with all cDNA samples examined. Thus, the NTT mRNA represents a rare gene product of the mammalian brain, whose expression is restricted to distinct CNS regions.

In conclusion, we have cloned cDNAs encoding a novel neurotransmitter transporter, NTT, which is likely to terminate synaptic transmission at selected synapses in the mammalian CNS. The precise nature of the transported transmitter is presently unclear, but

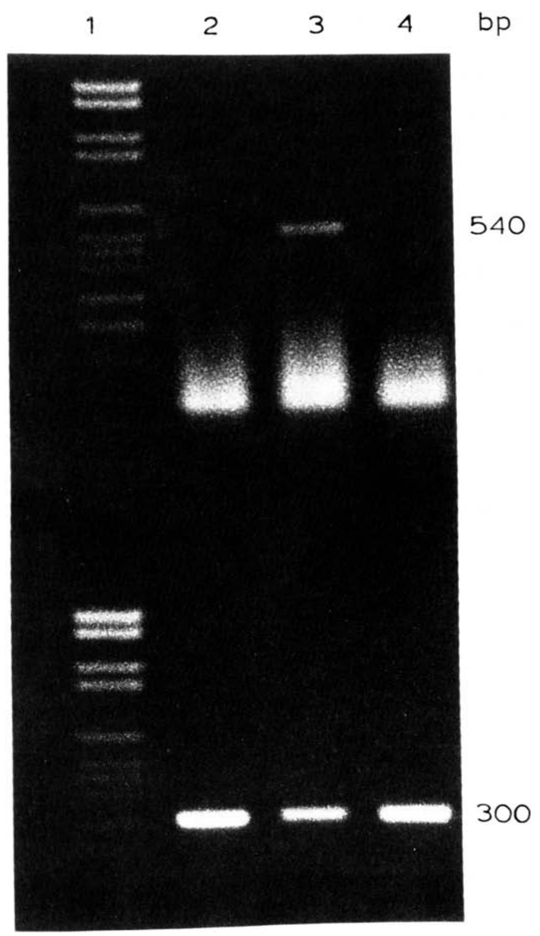

Fig. 3. PCR amplification of NTT and β-actin sequences from rat spinal cord, total brain and cerebellum cDNAs. Poly(A)$^+$ RNA was isolated and cDNAs synthesized using a kit from Boehringer (Mannheim). PCR was performed as described in section 2, except that the annealing temperature was 65°C. Products from brain (lane 2), spinal cord (lane 3) and cerebellum (lane 4) were resolved on a 1.5% agarose gel. Lane 1 contained DNA size markers (#6, Boehringer, Mannheim). Upper panel: NTT-specific products; lower panel: amplified β-actin sequences.

may include catecholamines, serotonin, or inhibitory and excitatory amino acids. Because of the low abundance of the corresponding transcripts in spinal cord and their apparent absence from rat brain and cerebellum, NTT is unlikely to constitute a glutamate, glycine or choline transporter. Expression studies in *Xenopus* oocytes or transfected cell lines should allow identification of the specific transmitter for NTT.

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