Volume 305, number 1, 31–36 FEBS 11184 June 1992

Primary structure and functional expression of a choline transporter expressed in the rat nervous system

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Received 29 April 1992

Synthesis of the neurotransmitter acetylcholine in cholinergic nerve terminals is regulated by a sodium-driven high-affinity choline uptake system in the plasma membrane. We have isolated cDNAs from rat spinal cord and brainstem which encode a choline transporter (CHOT1). The predicted protein shares considerable amino acid identity and several structural features including twelve putative transmembrane regions with other neurotransmitter transporters. Expression of in vitro transcribed CHOT1 RNA in *Xenopus* oocytes generated Na^+-dependent choline uptake, which was not seen in control oocytes. Amplification by polymerase chain reaction (PCR) revealed significant amounts of CHOT1 mRNA in brain, cerebellum, spinal cord and, to a lesser extent, heart, but only very low expression in lung, kidney and muscle.

Choline uptake; Neurotransmitter; Transporter; *Xenopus* oocyte; Nervous system; Rat

1. INTRODUCTION

Acetylcholine (ACh) was the first neurotransmitter to be identified in the nervous system [1]. After its release from presynaptic terminals, ACh binds to postsynaptic nicotinic and muscarinic ACh receptors, which activate distinct signal transduction mechanisms. Neurotransmission is terminated by dissociation of ACh from the receptor followed by its rapid enzymatic hydrolysis and reuptake of choline from the synaptic cleft into the presynaptic terminal (reviewed in [2–4]). Different investigators have shown that this reuptake process provides the major source of choline for the de novo synthesis of ACh [3,4]. Choline uptake therefore constitutes a rate-limiting step in ACh synthesis, and its modulation has been implicated in the control of synaptic efficacy [5–7].

[^{3}H]Choline-uptake studies with synaptosomes [8–10] and denervation experiments [11–13] indicate the presence of a Na^+-dependent high-affinity choline transport system at cholinergic nerve endings. Also, specific binding sites for the competitive inhibitor of choline uptake, hemicholinium-3 [8,14], have been demonstrated in brain tissue [15,16], and covalent attachment of this inhibitor as well as antibody labeling have been used to identify the insect neuronal choline transporter as a polypeptide of 80 kDa [17,18]. High affinity for choline and the requirement for Na^+ discriminate the neural choline uptake from an ubiquitous low-affinity choline transporter system, which provides the precursor for phospholipid synthesis in most types of cells [3,4,9].

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Recently, purification of a GABA transporter protein from rat brain [19] and molecular cloning of different cDNAs encoding Na^+-dependent mammalian neurotransmitter transporters have unraveled characteristic structural features of this family of membrane proteins [20]. The GABA (GAT1) [21,22], noradrenaline (NET1) [23], serotonin (SERT1) [24–26] and dopamine (DAT1) [27–30] transporters all share a common predicted transmembrane topology, with 12 putative membrane spanning segments, and significant amino acid identity (30–65%) along most of their sequence. We have exploited this conservation to search for cDNAs encoding novel neurotransmitter transporters by using the polymerase chain reaction (PCR) with degenerate oligonucleotides deduced from different regions of the GAT1 sequence [26]. Here we report that functional expression in *Xenopus* oocytes revealed one of our cDNA clones to encode a Na^+-dependent high-affinity choline transporter (CHOT1).

2. MATERIALS AND METHODS

2.1. *PCR amplification*

PCR was performed on oligo(dT)-primed cDNA isolated from rat spinal cord as described [26] using the following degenerate oligonucleotides complementary to the rat GAT1 cDNA [21]: 5'GGCTT(C/T)GT(C/G)AT(C/T)TT(C/T)TCCATC-3' (sense, corresponding to nucleotide positions 1003–1023); and 5'-GAA(C/G)CG(G/A)TT(G/C)AC(A/C)CC(A/G)TA(A/G)AACCA-3' (antisense, complementary to nucleotide positions 1417'1440). The ~440-bp amplification product was cleaved by the restriction enzyme *Ava*I, and residual undigested DNA was subcloned into pBluescript SK$^-$ and subjected to dideoxy sequencing [31] using synthetic primers.

2.2. *Isolation and sequence analysis of cDNA clones*

A rat spinal cord cDNA library constructed in λZAPII (7.5×10^5 pfu)

was screened with the ^{32}P-labelled Ava440 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. Inserts were excised from hybridization-positive phages as described in the Stratagene manual. Since no full-length clones were derived from this procedure, a randomly-primed λgt10 cDNA library prepared from rat brainstem (Clontech) was in addition screened with the ^{32}P-labelled Ava440 fragment. Phage DNA was prepared, and inserts were subcloned into pBluescript KS following standard procedures [32].

2.3. *Expression of CHOT1 in oocytes*

CHOT1 RNA was synthesized from the A15 cDNA subcloned in pBluescript KS by in vitro transcription using a commercial kit (Stratagene). Defolliculated oocytes of *Xenopus laevis* were prepared and injected with 50 nl of the CHOT-1 cRNA (1 μg/μl) as described [33]. After two days, individual oocytes were incubated in 200 μl of transport buffer 1 (TB1: 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.5) containing 0.25–1.0 μM [^{3}H]choline (specific activity 85 Ci/mmol; NEN, Dreieich, Germany). After 1 h at room temperature, the oocytes were washed 3 times in TB1, transferred into scintillation vials, solubilized by the addition of 100 μl of 10% (w/v) SDS, and radioactivity was determined by liquid scintillation counting. For evaluating specific CHOT1 mediated choline transport, values were corrected for tracer accumulation obtained with non-injected oocytes. All values represent the mean ±S.E.M. of 4–6 individual oocytes.

2.4. *Amplification of CHOT1 transcripts*

Poly(A)$^+$RNA was isolated from various rat tissues and reverse-transcribed into single-stranded cDNA using a commercial kit (Boehringer). PCR amplification of these cDNAs was performed as described [26] using two pairs of oligonucleotide primers corresponding to nucleotide positions 651–674 (sense), 1739–1763 (antisense), and 1227–1251 (antisense), of the CHOT1 cDNA. After 25 cycles, 5 μl of each reaction were separated on 1% agarose gels, blotted onto Nylon membranes, and specific amplification products were visualized by hybridization with radiolabeled CHOT1 cDNA probes. Parallel amplification of actin sequences was used to check the integrity and relative amounts of the different cDNA samples (see [26]).

3. RESULTS

3.1. *Structure of CHOT1 cDNA and protein*

In an attempt to clone putative transporter cDNAs from rat spinal cord, degenerate sense and antisense oligonucleotides covering selected sequences of the rat GAT1 cDNA [21] were designed to encompass a region which contains a unique *Ava*I site at nucleotide position 1105. Using these oligonucleotides for PCR amplification of spinal cord cDNA, a product of ~440 bp was obtained, which was subjected to *Ava*I digestion. In addition to the expected fragments of 335 bp and 102 bp corresponding to GAT1, uncleaved amplification product remained. Subcloning and sequence analysis of this DNA revealed a novel nucleotide sequence harbouring an open reading frame homologous to GAT1. We therefore used this fragment, termed Ava440, to screen a λZAPII rat spinal cord cDNA library under high stringency conditions. This resulted in the isolation of a partial 2691 bp clone, termed A40, which covered a large portion of the novel open reading frame and the 3′-untranslated sequence including a potential polyadenylation signal and a stretch of several A residues. To obtain the 5′-coding region, a λgt10 cDNA library prepared from rat brainstem was screened with a ~800 bp 5′ probe derived from the original A40 clone. This identified an overlapping cDNA (A15) of 2741 bp with an open reading frame of 1905 bp (Fig. 1). The initiation codon was assigned to an ATG at positions 637–639 on the basis of the Kozak initiation consensus sequence [34]. The open reading frame encodes a mature protein of 635 amino acids with a calculated molecular weight of 70,631 Da and a theoretical isoelectric point of 6.38.

The protein deduced from the A15 cDNA shares many structural features with known neurotransmitter transporter sequences. Twelve hydrophobic segments of 18–25 amino acid residues qualify as putative transmembrane domains (Fig. 2), and the lack of a N-terminal hydrophobic signal sequence suggests a transmembrane topology identical to that proposed for other transporters, with both N and C termini being located cytoplasmically. Two consensus sites for *N*-glycosylation are present in a large presumptive extracellular region between transmembrane segments III and IV (Fig. 1); this region diverges between the different transporters, but invariably contains *N*-glycosylation sites [20]. A further potential site for *N*-glycosylation is found in the most C-terminal extracellular loop between transmembrane domains XI and XII. Consensus sites for phosphorylation by protein kinase C and tyrosine kinase are located in the cytoplasmic N-terminal region and the short loop connecting transmembrane segments IV and V (Fig. 1).

Sequence comparison with GAT1, NET1, DAT1 and SERT1 (Fig. 2) revealed a high degree of conservation; corresponding amino acid identities are 49%, 42%, 41% and 37%, respectively. With isofunctional replacements being in addition considered, homology values increase to 64% for GAT1, 55% for NET1, 56% for DAT1 and 53% for SERT1. The highest sequence identity (51%; homology 65%), however, is found with the recently cloned betaine transporter, BGT1, from kidney, which transports also GABA and represents another member of the Na^+-dependent transporter superfamily [35]. A dendrogram derived from the sequence alignment (Fig. 2) places CHOT1 into a subfamily including GAT1 and BGT1, whereas the catecholamine transporters form a distinct branch that is closely related to the SERT1 protein. Most conserved residues and many isofunctional replacements are positioned within or adjacent to putative transmembrane segments. No conservation is seen in the highly charged N- and C-terminal tails.

3.2. *Functional expression in* Xenopus *oocytes*

In vitro transcribed A15 RNA was injected into *Xenopus* oocytes to test its putative neurotransmitter transporter function. After incubation with [^{3}H]choline, [^{3}H]glycine or [^{3}H]glutamate, significant accumulation

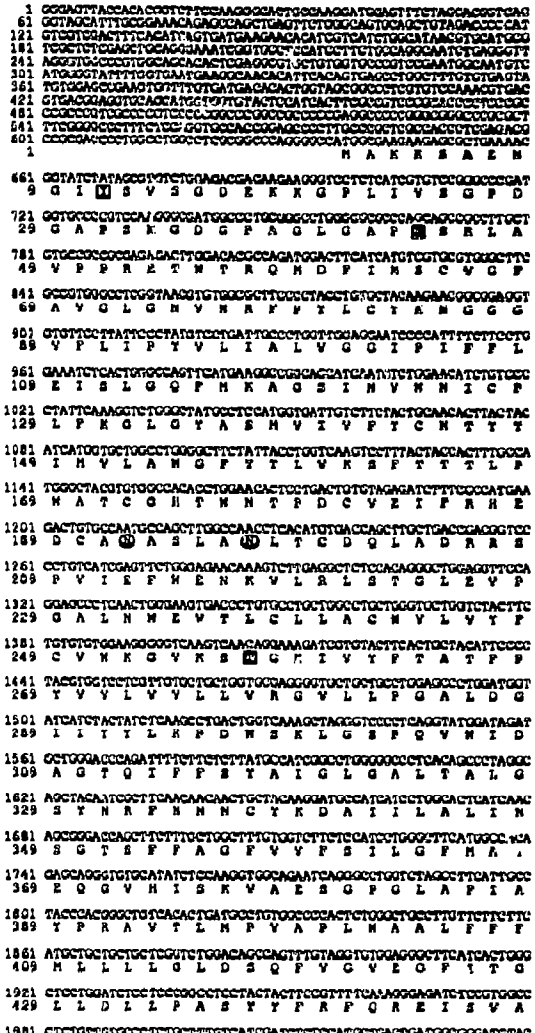


Fig. 1. Nucleotide and deduced amino acid sequences of the rat CHOT1 cDNA. A polyadenylation signal in the 3′-untranslated region of the nucleotide sequence is underlined, potential extracellular *N*-linked glycosylation sites are circled, and putative intracellular consensus sequences for phosphorylation by protein kinase C and tyrosine kinase are boxed. Numbering of nucleotides and amino acids is indicated on the left side of the figure. Clone A15 corresponds to nucleotides 1–2741, and clone A40 to nucleotides 1281–3972, of the DNA sequence.

of radioactivity exceeding that of non-injected control oocytes was only seen with [^{3}H]choline (Fig. 3A). At 1 μM [^{3}H]choline, this uptake was about 5–10 times higher as compared to control oocytes. Moreover, no uptake was seen when Na^+ was exchanged with Li^+, indicating that transport was Na^+-dependent. Competition studies with unlabelled substrate (Fig. 3B) revealed a maximal velocity (V_{max}) of 24.6 ±5.6 pmol/h of choline uptake per oocyte and a K_m of 9.6 ±3.9 μM (means of three independent experiments). The latter value is similar to those reported for the high-affinity choline uptake system present in rat brain synaptosomes [36]. Surprisingly, hemicholinium-3 had no significant effect on [^{3}H]choline uptake up to concentrations of 10^{-5} M (not shown). Control experiments showed that [^{3}H]choline uptake into non-injected oocytes differed from that seen upon cRNA expression in that (i) this uptake was not saturable up to 50 μM (Fig. 3B), and (ii) it showed no significant reduction upon substituting Na^+ by Li^+ (not shown). We hence conclude that the A15 cDNA encodes a Na^+-dependent high-affinity choline transporter protein, termed CHOT1.

3.3. *Tissue Distribution of CHOT1 transcripts*

Attempts to reveal CHOT1 transcripts in rat brain and spinal cord by Northern analysis were unsuccessful; presumably expression of the corresponding gene is low. PCR was therefore used to amplify CHOT1 sequences from poly(A)$^+$RNA preparations isolated from different rat tissues after reverse transcription into cDNA. As demonstrated in Fig. 4A, a ~1.1 kb PCR amplification product specific for CHOT1 was readily detected in developing (postnatal days 1 and 16) and adult spinal cord as well as in adult brain and cerebellum, but not in skeletal muscle, kidney, lung and heart. Using a second set of CHOT1-specific primers encompassing a shorter (600 bp) region of the RNA, significant amounts of CHOT1 amplification product were also revealed in heart (Fig. 4B). This result is unlikely to result from contamination by genomic DNA, but most likely reflects the presence of cholinergic cells in this organ. Indeed, autonomous innervation by cholinergic neurons is crucially implicated in the control of heart beat. Using a higher number of amplification cycles and/or longer autoradiographic exposure times, very low amounts of amplification product were also

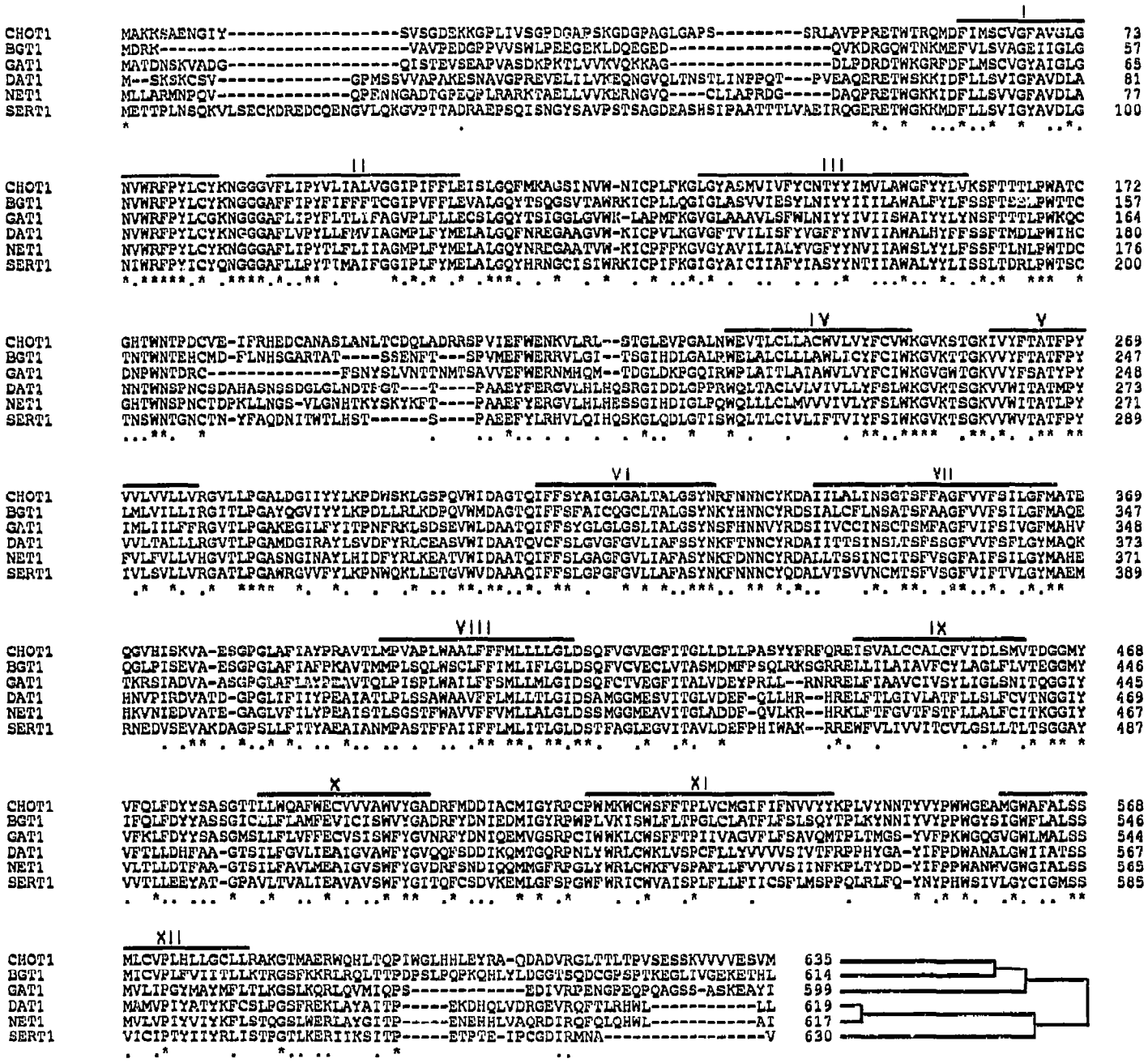
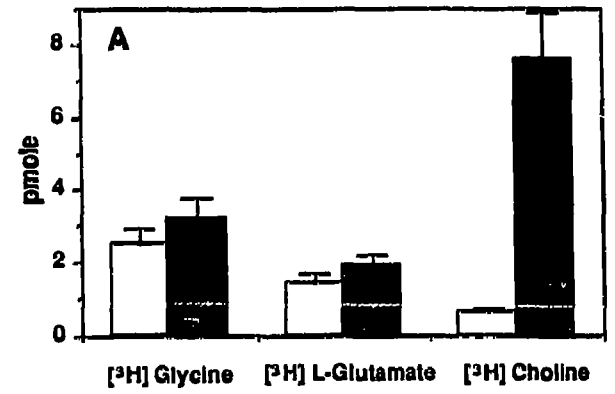


Fig. 2. Alignment of Na^+-dependent transporter proteins. Sequences are taken from the following references: BGT1, [35]; GAT1, [21]; NET1, [23]; DAT1, [28]; and SERT1, [26]. Amino acids identical in all transporters are marked by asterisks, and isofunctional residues shared by at least four proteins by a dot. Putative transmembrane regions are indicated by bars. Gaps were introduced to optimize identical sequence positions. A dendrogram at the C-terminal end of the sequences symbolizes the homology relationships between the individual transporters.

seen in muscle, lung and kidney (data not shown). This may be due to the presence of intramural parasympathetic ganglia. Control amplification with actin mRNA specific primers confirmed the integrity of the RNA preparations used for reverse transcription (Fig. 4C). In conclusion, these PCR data are consistent with a localization of CHOT1 transcripts in organs known to contain cholinergic neurons.

4. DISCUSSION

Different lines of evidence indicate that the CHOT1 cDNA described in this paper encodes a high-affinity choline transporter, which is highly expressed in the nervous system. First, the polypeptide sequence deduced from the single continuous open reading frame displays considerable homology to previously cloned



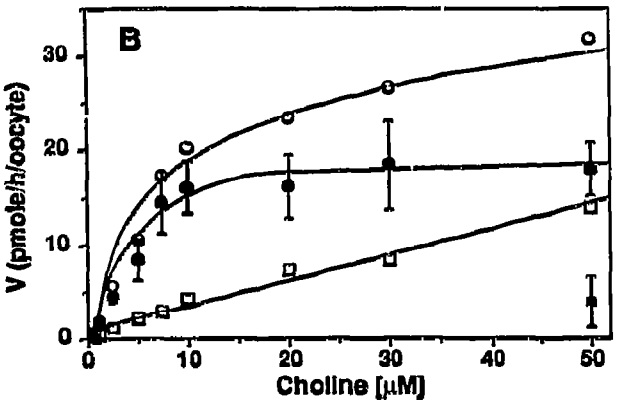


Fig. 3. Expression of CHOT1 in *Xenopus* oocytes. *Xenopus* oocytes were injected with synthetic A15 RNA, and [³H]choline uptake was determined after 2 days as described in section 2. A. Uptake of [³H]glycine, [³H]glutamate and [³H]choline in control (open bars) and injected (hatched bars) oocytes. B. Concentration dependence of [³H]choline uptake. Saturation characteristics were determined by the addition of different concentrations of unlabelled choline, and transport kinetic values calculated using the Eadie-Hofstee transformation [38]. Non-specific uptake was determined using non-injected oocytes (□); these values were subtracted from those obtained with RNA-injected cells (○) to yield CHOT1-specific uptake (●) data. (■), CHOT1-specific uptake in buffer, where Na^+ was substituted by Li^+. K_m and V_{max} values obtained in this particular experiment were 5.7 μM and 20.7 pmol/h per oocyte.

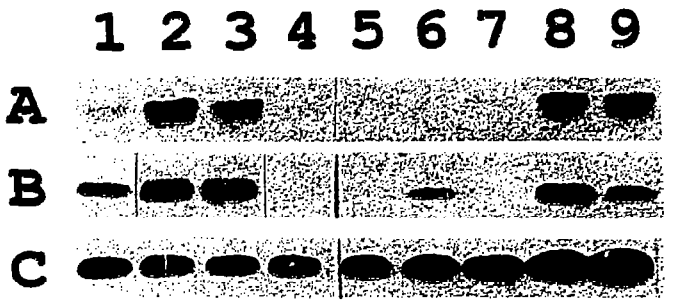


Fig. 4. Distribution of CHOT1 transcripts. PCR amplification with CHOT1-specific oligonucleotide primer combinations (A, fragment of 1112 bp; B, fragment of 600 bp) was performed in parallel using equal amounts of cDNA synthesized from poly(A)⁺RNA isolated from different rat tissues as detailed in section 2. Control amplifications were performed with a β actin-specific set of primer oligonucleotides (C). The following poly(A)⁺RNA preparations were used: lane 1, spinal cord, postnatal day 1; lane 2, spinal cord, postnatal day 16; lane 3, spinal cord; lane 4, skeletal muscle; lane 5, kidney; lane 6, heart; lane 7, lung; lane 8, total brain; and lane 9, cerebellum. Lanes 3–9, tissue from adult (6–8 week-old) rats.

neurotransmitter transporters, e.g. the GAT1, NET1, DAT1 and SERT1 proteins, as well as the betaine transporter BGT1. In particular, the BGT1 and GAT1 proteins show high sequence identities, suggesting that CHOT1 may belong to an amino acid-related transporter subfamily. Also, the transmembrane topology suggested by hydropathy analysis [37] closely resembles that assumed for the other transporter proteins [20]. CHOT1 thus clearly is a member of this integral membrane protein family. Second, heterologous expression of in vitro transcribed CHOT1 mRNA in *Xenopus* oocytes generated Na^+-dependent high-affinity [³H]choline uptake. Unexpectedly, this uptake was insensitive to the classical choline transport blocker hemicholinium-3 [8,14]; this may indicate that different choline transporter subtypes of distinct pharmacology may exist. Finally, PCR amplification of CHOT1 transcripts revealed expression of the corresponding gene in brain, spinal cord, cerebellum and, to a lesser extent, heart. This distribution of CHOT1 mRNA is consistent with the presence of cholinergic nerve cells in these tissues.

Different sequence features of CHOT1 and the other neurotransmitter transporter proteins enforce speculations about possible functional domains of these membrane proteins. Calculation of hydropathy values for the individual transmembrane segments [37] and analysis of their sequence conservation between the different transporters suggest that some regions may be particularly important in substrate binding and/or transport function. First, the putative transmembrane segment I shows a very high degree of conservation (Fig. 2), but only a low content of hydrophobic residues. This segment thus may be implicated in ion translocation and probably is centrally positioned in the assembled transporter. Segment IX in contrast is not well conserved and may have a specific function in substrate binding. Similarly, the large extracellular loop connecting segments III and IV is highly divergent, and thus may also participate in substrate recognition and/or translocation. Regions involved in Na^+ translocation have not been identified; however, a glutamate residue (Glu-490 in CHOT1) is absolutely conserved in transmembrane segment X. This side chain thus might constitute an intramembrane binding site for Na^+. Alternatively, it may stabilize transporter conformation by pairing with other intramembrane charged residues, e.g. a conserved arginine in segment I. Analysis of chimeric transporter constructs and site-directed mutagenesis experiments

should help to elucidate the precise role of these putative substrate and ion binding regions.

Acknowledgements: We thank A. Niehuis for expert technical assistance, Dr. H. Breer, B. Matzenbach and A. Ultsch for many helpful suggestions, Dr. Y. Maulet for providing the spinal cord cDNA library, Drs. D. Langosch and M. Duggan for critical reading of the manuscript, and S. Wartha for help with its preparation. This work was supported by Deutsche Forschungsgemeinschaft (Leibniz Programm) and Fonds der Chemischen Industrie.

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