Molecular cloning and characterization of a murine hemicholinium-3-sensitive choline transporter

S. Apparsundaram*, S. M. Ferguson† and R. D. Blakely‡

*Department of Pharmacology, Vanderbilt University Medical School, Nashville, TN 37232-6600, U.S.A., †Center for Molecular Neuroscience, Vanderbilt University Medical School, Nashville, TN 37232-6420, U.S.A., and ‡Graduate Neuroscience Program, Center for Molecular Neuroscience, Nashville, TN 37232-6420, U.S.A.

Abstract

In cholinergic neurons, a specific requirement for precursor choline in the biosynthesis of acetylcholine (ACh) is thought to be sustained by a presynaptic, hemicholinium-3 (HC-3)-sensitive choline transporter (CHT). This transporter exhibits micromolar affinity for choline and transport activity is Na⁺- and Cl⁻-dependent. Based on the sequence information available with the recent cloning of rat and human CHTs [Okuda, Haga, Kanai, Endou, Ishihara and Katsura (2000) Nat. Neurosci. 3,120-125; Apparsundaram, Ferguson, George Jr and Blakely (2000) Biochem. Biophys. Res. Commun. 276, 862-867; Okuda and Haga (2000) FEBS Lett. 484,92-97], we have identified a murine CHT orthologue (mCHT) by reverse transcriptase-PCR of spinal cord mRNA and confirmed this sequence using assembled mouse genomic DNA. Inferred splice junctions for mCHT exons are conserved with those of hCHT. The mCHT cDNA encodes a protein of 580 amino acids with 93% and 98% amino acid identity to human CHT and rat CHT1, respectively. Hydropathy analysis of the predicted amino acid sequence of mCHT indicates a protein containing 13 transmembrane domains (TMDs), with the N-terminus oriented extracellularly and the C-terminus oriented intracellularly. Northern blot analysis of mouse tissues reveals the expression of mCHT as a single transcript of ~5 kb with highest expression in regions that are rich in cholinergic cell bodies, e.g. the spinal cord, brainstem, mid-brain and striatum, whereas hybridization signals are absent in regions lacking cholinergic soma, e.g. the cerebellum and kidney.

Expression of mCHT in COS-7 cells results in high-affinity [3H]HC-3-binding sites (Kᵦ = 5 nM), and Na⁺- and Cl⁻-dependent HC-3-sensitive choline uptake (Kᵦ = 2 µM), assessed in resealed membrane vesicles. The availability of cloned, functional mCHT and its cognate genomic DNA should prove useful for studies of mCHT regulation and should open possibilities for evaluation of CHT dysfunction in murine models.

Introduction

Acetylcholine (ACh) is a major neurotransmitter in peripheral and central cholinergic neurons. While low-affinity choline uptake systems provide choline for metabolic needs, cholinergic neurons have a distinct high-affinity choline uptake (HACU) process that provides choline for the biosynthesis of ACh [1-4]. In mammalian cholinergic neurons, HACU is a Na⁺- and Cl⁻-dependent process, with a Kᵦ of 1-2 µM for choline and is potently inhibited by hemicholinium-3 (HC-3) [1,5,6]. HACU is thought to be rate limiting in the biosynthesis of ACh [7-9]. Selective blocking of HACU by HC-3 alters ACh synthesis and release, and thereby impairs cholinergic neurotransmission [10,11]. HACU activity is highly regulated by neuronal depolarization, second-messengers, hormones and neurotransmitters [12-20] but, owing largely to a lack of structural information about the HACU process, has received only a small amount of attention as a target for the therapeutic modulation of cholinergic neurotransmission. Similarly, our understanding of the role of the HACU process in disease states including Alzheimer's disease [21], Parkinson's disease [22], schizophrenia [23], Huntington's disease [24] and dysautonomia [25], and our ability to model such dysfunction in animal models is presently limited.

Recently, Okuda et al. achieved a breakthrough in studies of the HACU process with the report of a Caenorhabditis elegans choline transporter, CHO-1, and its rat orthologue, rCHT1 [26]. Based on the sequence information of rCHT1 and the information provided by Human

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Abbreviations used: ACh, acetylcholine; m/rhCHT, murine/rat human choline transporter; HACU, high-affinity choline uptake; HC-3, hemicholinium-3; TMD, transmembrane domain; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

*Present address: Department of Anatomy and Neurobiology, University of Kentucky Medical Center, Lexington, KY 40536-0098, U.S.A.

†To whom correspondence should be addressed (e-mail randy.blakely@mcmail.vanderbilt.edu).
structurally, these transporters are related to the sodium glucose co-transporter (SGLT) family of sodium-dependent glucose transporters rather than the Na+/Cl−-coupled co-transporters of the γ-aminobutyrate/noradrenaline (norepinephrine) transporter gene family, despite having similar ionic dependence. We now extend these studies with a report of a mouse CHT (mCHT) whose sequence, expression pattern and functional characteristics identify it as being likely to be involved in synaptic choline transport at murine cholinergic synapses.

**Materials and methods**

**Reverse transcriptase (RT)-PCR of mCHT cDNA**

We designed degenerate oligonucleotides for the amplification of the full open reading frame of mCHT from mouse spinal cord RNA, based on the sequence information of rCHT1 and the information provided by the human genomic sequence database (GenBank accession number AC009963). The sense primer RB892 (5'-CTGGATCCAAATGGAATCCTTCCATGTA/GG AAGG-3') overlaps the putative start codon (underlined) and extends by 21 nucleotides on the 5′-end and seven nucleotides on the 3′-end. The antisense primer RB893 (5'-GACTCGAGGTCAC/TTTGTAACA/TGTATCCTCACGTCCC-3') begins 15 bases 3′ of the putative stop codon (underlined). Mouse spinal cord total RNA was isolated using TRIZOL reagent (Life Technologies) and the RT-PCR amplification was carried out using the Prostar Ultra HF RT-PCR system (Stratagene) following the manufacturer’s protocol. Briefly, COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal bovine serum (HyClone) and 100 μg/ml penicillin, at a density of 5 × 10^6 cells in 150 mm culture dishes (Falcon, Lincoln Park, NJ, U.S.A.). Choline transport was determined in resealed membrane vesicles derived from mCHT-expressing COS-7 cells. Vesicles prepared from vector-transfected cells were used as controls. For the preparation of membrane vesicles, cell monolayers were washed with 30 ml of HTE buffer (10 mM HEPES, 10 mM Tris base, 1 mM potassium EDTA, pH 7.4) and harvested in 10 ml of homogenization buffer (0.32 M sucrose in HTE buffer). Cells were homogenized at 20000 rev./min for 25 s using a Polytron tissue homogenizer (Brinkman). The homogenate was centrifuged at 100000 g for 45 min at 4°C. The membrane pellet was resuspended in 2 ml of...
HTE buffer, the suspension was rotated gently at 4°C for 45 min. Membranes were centrifuged (27000g) for 20 min at 4°C and the resulting pellet was suspended in 2 ml of Na+/Cl–-free HTE buffer. Protein content of the suspension was determined using the Bradford method and 50 μg of the vesicle suspension (1 μg/μl) was transferred to each tube to be assayed in triplicate. [3H]Choline transport was initiated by the addition of [3H]choline (83 Ci/mmol, Amersham), such that the final concentration of labelled choline was 20 nM. Unless otherwise stated, in all transport assays ion gradients were established using an external NaCl concentration of 150 mM. After a 5 min incubation at room temperature with labelled choline, vesicles were filtered rapidly through Whatman GF/B filters soaked in 0.3% polyethyleneimine in HTE buffer. The filters were washed rapidly five times with 3 ml of ice-cold HTE buffer and the accumulated radioactivity quantified using scintillation spectrometry (Packard). In experiments designed to determine the ion-dependence of choline transport, either Na+ or Cl– was replaced with equimolar quantities of Li+ or isoethionate, respectively. Non-specific uptake was quantified by determining uptake in the presence of 1 μM HC-3 and this was subtracted from the total uptake to yield specific transport.

Figure 1

Alignment of amino acid sequence of high-affinity choline transporters

Alignment of mCHT with species orthologues was performed using version 1.6.3 of Lasergene software. Residues matching mCHT sequences are shaded (black). Residues spanning putative TMDs inferred from hydropathy analysis are represented by lines drawn above the sequences. LChCoT, Limulus polyphemus CHT; CHO-1, Caenorhabditis elegans CHT.
For radioligand binding assays, membrane fractions were prepared as described earlier and 50 μg of the membrane suspension (1 μg/μl) was transferred to each tube. Binding assays were initiated by the addition of [3H]HC-3 (127 Ci/mmol; NEN Life Science Products) to a final concentration of 10 nM. After incubation at room temperature for 1 h, membranes were rapidly filtered, and the filters processed for quantification of the accumulated labels, as described earlier. Unless otherwise indicated, all binding assays were performed in the presence of 150 mM NaCl in HTE buffer. Non-specific binding was quantified by determining [3H]HC-3 binding in the pcDNA3-transfected COS-7 cells.

Results and discussion

Cloning of mCHT cDNA

We used degenerate oligonucleotides in RT-PCR to amplify a 1780 bp product from mouse spinal cord RNA. The PCR product comprises what we expect is a full-length open reading frame for mCHT and exhibits a 98% nucleotide identity to rCHT1. BLAST analysis of the Celera Discovery System™ v3.01 mouse genomic DNA assembly using our mCHT cDNA yielded an identical sequence for the coding exons of mCHT and conserved intron/exon boundaries with the human CHT gene. The mCHT cDNA (GenBank accession number AF276872) encodes a protein of 580 amino acids and has a predicted molecular mass of 63 kDa with a calculated isoelectric point of 4.9 (Figure 1). Hydropathy analysis of mCHT amino acid sequence indicates the presence of 13 transmembrane domains (TMDs) and a short extracellular N-terminus and large intracellular C-terminus, consistent with our predicted model for the secondary structure of hCHT and the recently reported model for *Limulus* ChCoT [27].

There are three canonical sites for N-linked glycosylation with Aspas and Asp17 oriented extracellularly. Canonical sites for phosphorylation by protein kinase C are present on the putative cytoplasmic domain between TMD-9 and TMD-10 (Ser567 and Ser573), as well as in the C-terminus (Thr558). Canonical sites for protein kinase A are also present in the cytoplasmic region (Ser580) and at the C-terminus (Ser582 and Ser585). Sequence alignments reveal that the mCHT protein exhibits 93%, 98% amino acid identity with hCHT and rCHT1 respectively, and 50%, 46% amino acid identity with *C. elegans* CHO-1 and *L. polyphemus* ChCoT, respectively (Figure 1). Overall there is 39% amino acid identity among all the known choline transporters and a 93% identity between mCHT, hCHT and rCHT1. Conserved residues are likely to play a role in choline recognition or in conserved aspects of substrate translocation. Together, these proteins constitute a new family of proteins that are distantly related to the solute carrier superfamily (SLC) 5A family of solute carriers, which includes the Na⁺-coupled glucose transporters SGLT1 (25% amino acid identity) and Na⁺/I⁻ symporter (21% amino acid identity) [30,31].

CHT mRNA expression

We performed a Northern analysis using a 547 bp cDNA fragment corresponding to the most 3' region of the mCHT coding sequence as a probe to ascertain the expression pattern and possible mRNA heterogeneity of mCHT. Consistent with the distribution of cholinergic cell bodies in the mouse central nervous system [32,33], our Northern blot analysis revealed a restricted regional distribution of mCHT mRNA as a single transcript of approx. 5 kb in the spinal cord, brainstem, midbrain and striatum (Figure 2). As yet, we have no evidence for CHT splice variants, though further analyses are warranted. No hybridization

![Figure 2](image-url)
was evident in the cerebellum, kidney, rat pheochromocytoma PC12 cells and human neuroblastoma LA-N-2 cells, despite evidence of control hybridization with the GAPDH probe. Preliminary studies with CHT fusion protein and peptide-directed antibodies [34] also support a localization of CHT protein in cholinergic soma and terminals.

**Choline transport and HC-3 binding assays**

Initial transport studies with intact, transfected cells revealed that CHT-induced choline transport activity was too low to permit reliable kinetic analyses or pharmacological characterization. We suspected that as with our previous studies of hCHT [27] functional mCHT protein was probably sequestered in non-trafficked, intracellular membranes. In order to access the intracellular pool of CHTs, we evaluated CHT transport in resealed membrane vesicles prepared from total cell membranes. Using this strategy, we readily detected a specific accumulation of labelled choline that was absent from vesicles prepared from vector-transfected COS-7 cells. Furthermore, the presence of 1 μM HC-3 reduced choline uptake to levels seen in vector-transfected COS-7 membrane vesicles. The specific uptake of labelled choline was found to have single-site saturation kinetics, with a $K_m$ of 2 μM (Figure 3A). As with hCHT [27,28], replacement of either Na+ with Li+ or Cl− with isoethionate reduced choline uptake to levels seen in vector-transfected cells (data not

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**Figure 3**

HACU and HC-3 binding sites are evident following transient expression of mCHT in COS-7 cells

(A) Uptake of choline in resealed membrane vesicles derived from mCHT transfected COS-7 cells is saturable ($K_m = 2$ μM). The inset is a Eadie–Hofstee transformation of mCHT-mediated choline uptake in resealed membrane vesicles. (B) Unlabelled HC-3, choline and ACh dose-dependently inhibit [3H]choline uptake in resealed membrane vesicles of COS-7 cells. Unlabelled HC-3, choline and acetylcholine were co-incubated with labelled choline. Acetylcholine was used in the presence of 10 μM physostigmine to inhibit cholinesterase. [3H]Choline uptake determined in the presence of 1 μM HC-3 is defined as non-specific uptake. (C) Binding of [3H]HC-3 in membrane fractions of mCHT-expressing COS-7 cells is saturable and is of high-affinity. Binding obtained in membrane fractions from pCDNA3-transfected cells were subtracted to define specific CHT-mediated [3H]HC-3 binding. Insets are Scatchard transformations of HC-3 binding. (D) Unlabelled HC-3, choline and acetylcholine dose-dependently inhibit [3H]HC-3 binding in membrane fractions of COS-7 cells. In A–D, data are presented as mean±S.E.M. of triplicate measurements.
shown). Co-application of unlabelled HC-3 and choline inhibited mCHT-mediated choline uptake in a dose-dependent fashion (Figure 3B). The IC$_{50}$ for HC-3 and choline at mCHT were 5 and 125 nM, respectively. ACh combined with choline-esterase blockade also inhibited CHT-mediated choline uptake (IC$_{50} = 570 \mu$M).

We also determined the binding characteristics of the selective CHT antagonist HC-3 [35]. Compared with vector-transfected cells, expression of mCHT cDNA in COS-7 cells produced a marked increase in [$^{3}H$]HC-3 binding in membrane fractions. Like choline transport, binding of [$^{3}H$]HC-3 is saturable and has high affinity (Figure 3C). Scatchard transformation of [$^{3}H$]HC-3 binding in mCHT-expressing membranes indicated single-site kinetics with an equilibrium dissociation constant (K$_{d}$) of 5 nM. Replacement of Na$^{+}$ with Li$^{+}$ or Cl$^{-}$ with isoethionate reduced [$^{3}H$]HC-3 binding in mCHT-expressing cells to levels seen in vector-transfected cells (data not shown). Unlabelled HC-3, choline and ACh (in the presence of physostigmine) inhibited [$^{3}H$]HC-3 binding in mCHT-transfected COS-7 cell membranes in a dose-dependent fashion. The K$_{i}$ values for HC-3, choline and ACh in inhibiting [$^{3}H$]HC-3 binding were 5 nM, 22 µM and 433 µM, respectively (Figure 3D).

In summary, our cloning and functional expression studies, along with the pattern of mRNA expression as revealed by Northern hybridization, suggest that mCHT encodes the protein that supports HACLT in murine cholinergic neurons. The availability of mCHT and access to its genomic DNA should permit the generation of models useful for the study of high-affinity choline transport function and regulation in vitro and in vivo.

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