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Human Neurons Express the Polyspecific Cation Transporter hOCT2, Which Translocates Monoamine Neurotransmitters, Amantadine, and Memantine

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ABSTRACT

Recently, we cloned the human cation transporter hOCT2, a member of a new family of polyspecific transporters from kidney, and demonstrated electrogenic uptake of tetraethylammonium, choline, N^1-methylnicotinamide, and 1-methyl-4-phenylpyridinium. Using polymerase chain reaction amplification, cDNA sequencing, *in situ* hybridization, and immunohistochemistry, we now show that hOCT2 message and protein are expressed in neurons of the cerebral cortex and in various subcortical nuclei. In *Xenopus laevis* oocytes expressing hOCT2, electrogenic transport of norepinephrine, histamine, dopamine, serotonin, and the antiparkinsonian drugs memantine and amantadine was demonstrated by tracer influx, tracer efflux, electrical measurements, or a combination. Apparent K_m values of 1.9 ± 0.6 mM (norepinephrine), 1.3 ± 0.3 mM (histamine), 0.39 ± 0.16 mM (dopamine), 80 ± 20 μM (serotonin), 34 ± 5 μM (memantine), and 27 ± 3 μM (amantadine) were estimated. Measurement of *trans*-effects in depolarized oocytes and human embryonic kidney cells expressing hOCT2 suggests that there were different rates and specificities for cation influx and efflux. The hypothesis is raised that hOCT2 plays a physiological role in the central nervous system by regulating interstitial concentrations of monoamine neurotransmitters that have evaded high affinity uptake mechanisms. We show that amantadine does not interact with the expressed human Na^+/Cl^- dopamine cotransporter. However, concentrations of amantadine that are effective for the treatment of Parkinson's disease may increase the interstitial concentrations of dopamine and other aminergic neurotransmitters by competitive inhibition of hOCT2.

In 1994, we cloned from rat kidney the cation transporter rOCT1 and demonstrated that it translocates small organic cations with diverse structures, including choline and some monoamine neurotransmitters, into epithelial cells in an electrogenic fashion (Gründemann *et al*., 1994; Busch *et al*., 1996a, 1996b; Koepsell, 1998). In distinction from other cloned Na^+/Cl^--dependent transporters for norepinephrine, dopamine, and serotonin (Rudnick and Clark, 1993), the OCT-mediated transport of monoamines and other cations is independent of a transmembrane Na^+ gradient. rOCT1 was the first member of a rapidly growing transporter family that contains highly homologous electrogenic cation transporters (Busch *et al*., 1996b; Okuda *et al*., 1996; Schweifer and Barlow, 1996; Gorboulev *et al*., 1997; Gründemann *et al*., 1997; Zhang *et al*., 1997a, 1997b, Terashita *et al*., 1998) and the polyspecific organic anion transporter OAT1 (Sekine *et al*., 1997). The OCT family belongs to a superfamily that includes multidrug resistance proteins, facilitative diffusion systems, and proton symporters (Marger and Saier, 1993). Recently, the highly homologous OCT subtype rOCT2 was isolated from rat kidney (Busch *et al*., 1996b; Okuda *et al*., 1996), and homologous transporters were identified from pig (pOCT2; Gründemann *et al*., 1997) and human (hOCT2; Gorboulev *et al*., 1997). Significant species differences in tissue distribution and function of the OCT1 and OCT2 transporters were observed. For example, recent data suggest that in different species, OCT2 transporters may be localized in luminal or basal membranes of renal epithelial cells (Gorboulev *et al*., 1997; Karbach U and Koepsell H, unpublished observations). Using PCRs, mRNAs with homology to *rOCT2* and *hOCT2* were detected in brain of rat and human (Gorboulev *et al*., 1997, Gründemann *et al*., 1997). Because Na^+-independent

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ABBREVIATIONS: PCR, polymerase chain reaction; PBS, phosphate-buffered saline; SSC, standard saline citrate; MPP, 1-methyl-4-phenylpyridinium; hDAT, human Na^+/Cl^- dopamine cotransporter; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEK, human embryonic kidney; TEA, tetraethylammonium; MES, 3-(*N*-morpholino)propanesulfonic acid.

transport of choline, thiamine, or norepinephrine has been detected in brain and glial cells (Yamamura and Snyder, 1973; Paterson and Hertz, 1989; Streich *et al*., 1996), we hypothesized that the cation transporters of the OCT family could serve these functions. In the current study, we show that the polyspecific organic cation transporter hOCT2 is transcribed in neurons of human brain and transports the monoamine neurotransmitters dopamine, norepinephrine, serotonin, and histamine and the antiparkinsonian drugs amantadine and memantine, which are known to increase interstitial monoamine neurotransmitter concentrations.

Experimental Procedures

PCRs, DNA sequencing, and generation of cRNA probes. Total RNA was isolated from a small piece of human frontal cortex obtained during removal of a subcortical tumor, as approved by the local ethics committee (Gorboulev *et al*., 1997). To remove traces of genomic DNA, the RNA was treated with RNase-free DNase (Stratagene, Heidelberg, Germany). cDNA was reverse transcribed and subjected to PCR analysis (RT-PCR) using *hOCT1*- and *hOCT2*-specific primers and the Expand Long Template PCR System (Boehringer-Mannheim, Mannheim, Germany). For amplification of *hOCT1*, two primers from 5′- and 3′-noncoding regions of *hOCT1* mRNA were used: K12$^+$, 5′-CAT GAG CAT GCT GAG CCA T-3′ (positions 53–71) and K4$^-$, 5′-GGG TAG GCA AGT ATG AGG-3′ (positions 1828–1845). For *hOCT2*, a series of primers were used, including two primers from 5′- and 3′-noncoding regions of *hOCT2* mRNA: K11$^+$, 5′-GCC CTC CTG CCT GCA GGA T-3′ (positions 125–143) and K7$^-$, 5′-TAG ATG CTC CTC TCC CAA C-3′ (positions 2188–2206). The primers K11$^+$ and K7$^-$ flank a region including intronic DNA of >1.5 kbp (data not shown). PCR was performed according to the manufacturer′s recommendations with an annealing temperature of 56°. Amplification fragments were isolated from agarose gel and sequenced using Sequitherm cycle sequencing kit (Biozym Diagnostik, Oldendorf, Germany). For the hybridization of Northern blots and for *in situ* hybridization, we used the same *hOCT2*-specific cRNA antisense and sense probes as described previously (Gorboulev *et al*., 1997). For the synthesis of the 430-bp probe, a cDNA fragment of *hOCT2* (positions 1776–2206) with low homology to *hOCT1* was amplified by RT-PCR and subcloned into pBluescript II SK(−). The plasmid was linearized with *Bam*HI (sense) and *Eco*RI (antisense) restriction enzymes, and the respective cRNAs were synthesized using T7 and T3 RNA polymerase in the presence of digoxigenin-labeled rUTP.

Northern blots and *in situ* hybridization. Northern blots were performed with poly(A)$^+$ RNA from different areas of human brain that was obtained from Clontech (Heidelberg, Germany). Then, 2 μg of RNA was applied per lane to a denaturating formaldehyde 1.2% agarose gel, transferred to a charged modified nylon membrane, and fixed by UV irradiation. Hybridization of the Northern blot was performed at 69° using the DTG-Easy-Hyb kit (Boehringer-Mannheim) and a probe concentration of 100 ng/ml. The hybridization signal was analyzed with the DIG-detection kit (Boehringer-Mannheim) with an alkaline phosphatase-coupled anti-digoxigenin antibody. Gel loading was controlled by hybridization with a 400-bp digoxigenin-labeled antisense cRNA probe of β-actin.

The *in situ* hybridization was performed with human tissue that was taken 4–10 hr *post mortem* and rapidly frozen in liquid nitrogen. Next, 8-μm cryostat sections of human brain were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline, incubated 10 min with 0.1 M HCl, and hybridized by 16-hr incubation at 50° in 8 mM triethanolamine-HCl, pH 7.6, containing 0.25 M NaCl, 4 mM EDTA, 5% (w/v) dextran sulfate, 45% (w/v) deionized formamide, 0.9 mg/ml yeast tRNA, and 1 μg/ml concentration of the digoxigenin-labeled cRNA probes. The sections were incubated for 30 min (65°) with 2× SSC, for 30 min (70°) with 1× SSC, and for 30 min (37°) with 0.1 M Tris·HCl, pH 7.5, containing 0.4 M NaCl, 50 μM EDTA, and 20 μg/ml RNase A. After an additional 30-min (50°) incubation with 1× SSC and 15 min (20°) with 1% (w/v) blocking reagent (Boehringer-Mannheim), the sections were incubated with Fab′ fragment of alkaline phosphatase-conjugated anti-digoxigenin antibody from goat and visualized by incubation with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Gorboulev *et al*., 1997).

Immunohistochemistry. An antibody was raised in rabbits against a peptide representing residues 317–332 of hOCT2. The peptide was coupled to ovalbumin and the immunization was performed as described (Poppe *et al*., 1997). Next, 5-μm-thick cryosections were fixed with 4% (w/v) paraformaldehyde dissolved in 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.6 mM KH_2PO_4 (PBS) and blocked with PBS containing 2% (w/v) skim milk powder and 0.05% (w/v) Triton X-100 (PBS-MT). For the antibody reaction, the sections were incubated for 20 hr (4°) with the antiserum, which was diluted 1:200 in PBS-MT. After washing with PBS-MT, the sections were incubated for 2 hr at room temperature with peroxidase-labeled goat anti-rabbit IgG, which was diluted 1:50 in PBS-MT. The immune reaction was visualized using diaminobenzidine (Graham and Karnovsky, 1966). The specificity of the antibody reaction was verified by negative controls on parallel sections. The controls were incubated with preimmune serum or with the antiserum that had been blocked with the antigenic peptide.

Expression of hOCT2 in oocytes of *Xenopus laevis* and transport measurements. The cDNA of *hOCT2* was subcloned into a pOG2 vector containing untranslated regions of the *Xenopus*/β-globin gene and linearized with *Not*I, and cRNA was synthesized (Gorboulev *et al*., 1997). *X. laevis* oocytes were collected, defolliculated, and stored several hours in Ori buffer [5 mM 3-(*N*-morpholino)propanesulfonic acid-NaOH, pH 7.4, 100 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$] containing 50 mg/liter gentamycin. Oocytes then were injected with 50 nl of water/oocyte with or without 10 ng of hOCT2 cRNA or hDAT cRNA and incubated 2–3 days at 19° in Ori buffer containing 50 mg/liter gentamycin. To preload the oocytes with organic cations, the final 12 hr of incubation were performed in the presence of these cations. The same results were obtained when the oocytes were preloaded by injecting 50 nl of 10-fold concentrated organic cation solutions. After washing of the oocytes at 0° in Ori buffer or K oocyte buffer [5 mM MES-KOH, pH 7.4, 100 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$] influx measurements with radioactively labeled cations were performed after incubation of the oocytes at 19° in Ori or K oocyte buffer. Median ± standard error values were calculated from 8–10 oocytes or oocyte pairs. For efflux measurements, the oocytes were injected with 50 nl of [^{3}H]MPP (0.1 pmol), washed at 0°, and transferred to test tubes containing Ori or K oocyte buffer (19°) without or with organic cations. In Fig. 5c, the [^{3}H]MPP efflux at each experimental condition was measured in three oocytes after 1-, 2-, 3-, 4-, and 5-min incubations, and initial efflux rates were calculated from monoexponential curves, which were fitted to the data (Busch *et al*. 1996). In Fig. 9a, the initial efflux rates were calculated from the efflux between 10- and 70-sec incubations.

Electrophysiology. For electrical measurements, oocytes were superfused with 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES-Tris, pH 7.4, or with 98 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES-Tris, pH 7.4 (~3 ml/min, 22–24°). Measurements of the membrane potential and two-electrode voltage-clamp recordings were performed as described previously (Busch *et al*., 1996b; Nagel *et al*., 1997). The cation-induced change of the membrane potential and the size of cation-induced currents varied significantly, depending on the batch of oocytes. Data are shown for sets of experiments that were each obtained on the same day. The experiments were repeated with two or three batches of oocytes, and qualitatively similar results were obtained. For the determination of current-voltage relations, steady state current was measured during the last 100 msec of 500-msec rectangular voltage pulses to different potentials. The pulses were applied from a holding potential of −50 mV at a frequency of 0.4 Hz. For measurements of membrane poten-

tials, HEK 293 cells were grown on glass coverslips near confluence and mounted as the bottom of a perfusion chamber on the stage of an inverted microscope. The cells were perfused constantly with buffers without and with K^+, and the membrane voltages were measured using the slow-whole cell patch-clamp method. To gain electrical access to the cells, the pipette solutions contained 100 mg/liter nystatin.

Expression of *hOCT2* in HEK 293 cells and transport measurements. *hOCT2* was subcloned into the expression vector pRc-CMV (InVitrogen, Leek, The Netherlands), and the construct was used to transfect HEK 293 cells (CRL-1573; American Type Culture Collection, Rockville, MD), which were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum (Sigma, Deisenhofen, Germany). The transfected cells were selected for neomycin resistance by growing them in the presence of increasing geneticin (G418) concentrations (0.2–0.8 mg/ml), and single constantly transfected clones were isolated. The transport measurements were performed with suspended cells derived from a single clone. When the cells became confluent 4–5 days after passage, they were washed with PBS and suspended by shaking. The cells were collected by 10-min centrifugation at 1000 × *g* and suspended at 37° in PBS or in K buffer (139.7 mM KCl, 8 mM K_2HPO_4, and 1.6 mM KH_2PO_4, pH 7.4). In some experiments, the cells were preloaded with cations by incubating them for 30 min at 37° with PBS or K buffer containing radioactively labeled or nonradioactive organic cations. After this time period, equilibrium was reached (Fig. 10a). Then, the cells were spun down at 1000 × *g*, suspended in ice-cold PBS or K buffer, and washed twice at 0° with the respective buffer. For uptake measurements, preloaded or nonpreloaded cells were warmed (40 sec at 37°) and suspended in PBS or K buffer (37°) that contained different concentrations of [^{3}H]MPP, [^{3}H]dopamine, or [^{3}H]choline with or without 200 μM cyanine 863 (Busch *et al.*, 1996a). Efflux measurements from cells preloaded with radioactive cations were performed by incubating 20 μl of ice-cold cell suspensions with 200 μl of PBS or K buffer (37°). Uptake and efflux reactions were stopped with ice-cold PBS containing 100 μM quinine.

Materials. [^{3}H]Dopamine (1.8 TBq/mmol), [^{3}H]choline (2.6 TBq/mmol), [^{3}H]serotonin (0.7 TBq/mmol), [^{3}H]norepinephrine (0.4 TBq/mmol), and [^{3}H]histamine (1.9 TBq/mmol) were obtained from Amersham Buchler (Braunschweig, Germany). 1-[^{3}H]methyl-4-phenylpyridinium (3.1 TBq/mmol) was from Du Pont de Nemours (Dreieich, Germany). [^{14}C]Memantine (5.9 GBq/mmol) was a kind gift of Merz (Frankfurt, Germany). Amantadine, memantine, and peroxidase-coupled goat anti-rabbit IgG antiserum were purchased from Sigma. All other chemicals were obtained as described previously (Busch *et al.*, 1996b).

Results

The transcription of *hOCT1* and *hOCT2* in human brain was investigated. Previously, we amplified a cDNA fragment from human brain that was identical to nucleotides 1648–1845 of *hOCT1* (Gorboulev *et al.* 1997). Using 5′ and 3′ primers from noncoding cDNA regions of *hOCT1* for RT-PCR experiments (see Experimental Procedures), the full-length clone could be amplified from liver but not from brain. This indicates that *hOCT1* is not transcribed in human brain and that the previously detected carboxyl-terminal cDNA fragment belongs to an unknown gene product with a domain identical to *hOCT1*. Fig. 1 shows a Northern blot with mRNAs of different brain areas that was hybridized with an *hOCT2*-specific cRNA probe. In brain, much smaller hybridization signals were obtained than in the kidney (<5%, data not shown). In brain, the main hybridization was observed at ~4.4 kb, but two distinct hybridization bands at ~2.5 and 4–4.5 kb were obtained in kidney (Gorboulev *et al.*, 1997). The hybridization in hippocampus and various subcortical nuclei such as thalamus, nucleus subthalamicus, nucleus caudatus, and nucleus amygdaloideus was stronger than that in substantia nigra (Fig. 1). To verify whether *hOCT2*, rather than a highly homologous gene, is expressed in human brain, PCRs were performed with reverse-transcribed mRNA from human frontal cortex. A series of overlapping *hOCT2*-specific primers were used, including the primers $K11^+$ and $K7^-$ (see Experimental Procedures), which were derived from the 5′- and 3′- noncoding region of *hOCT2* mRNA, respectively. DNA sequencing of the overlapping PCR fragments showed that full-length mRNA of *hOCT2* is transcribed in human brain. The cDNA sequence was identical with that of *hOCT2* from kidney (Gorboulev *et al.*, 1997).

Previously, an Na^+-independent polyspecific organic cation transport system was characterized in the myocardial, smooth muscle, or glandular cells that translocates norepinephrine and was called extraneuronal norepinephrine uptake$_2$ system (Iversen, 1967; Trendelenburg, 1988). Because recent uptake studies with the human glioma cell line SK-MG-1 suggested that this norepinephrine uptake$_2$ system also is expressed in brain (Streich *et al.*, 1996), we performed RT-PCR experiments to elucidate whether *hOCT1* and *hOCT2* are transcribed in SK-MG-1 cells. *hOCT2* cDNA could not be amplified. With primers from the 5′- and 3′-noncoding regions of *hOCT1*, a 2.5-kb fragment with partial sequence identity to *hOCT1* rather than the expected 1.8-kb fragment of *hOCT1* was amplified. This suggests that a splice variant of *hOCT1* is expressed in glial cells that could be identical to the norepinephrine uptake$_2$ system.

To determine the cellular localization of hOCT2 message, *in situ* hybridization was performed with an *hOCT2*-specific cRNA probe using sections of human cerebral cortex and

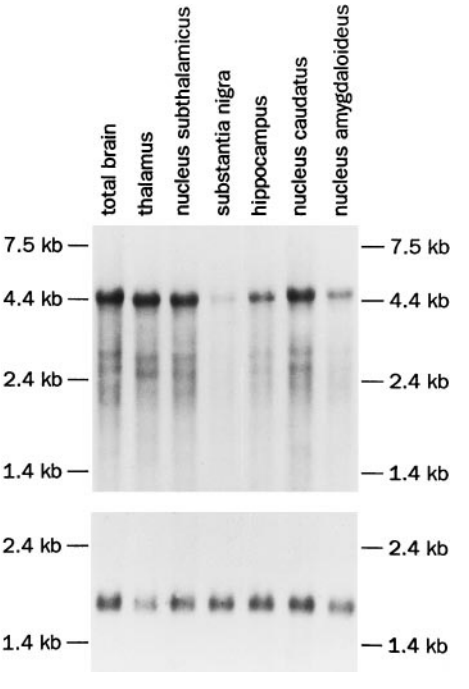

Fig. 1. Distribution of hOCT2 in brain of the human analyzed by Northern blotting. *Top*, 2 μg/lane of poly(A)$^+$ mRNA from different brain areas was separated by agarose gel electrophoresis and hybridized under high stringency conditions with a DIG-labeled cRNA probe specific for *hOCT2*. *Bottom*, intactness of the mRNAs in the different lanes was controlled by hybridization with cRNA of β-actin.

hippocampus. Fig. 2 shows that *hOCT2* is transcribed in pyramidal cells of cerebral cortex (area 18) and in pyramidal cells of hippocampus. The mRNA of *hOCT2* was detected in the somata and in dendrites of neurons (Fig. 2e). Subsequently, an hOCT2-specific peptide antibody was raised to examine hOCT2 protein expression in hippocampus. Using this antibody, the same localization of hOCT2 in human kidney was observed as described previously (Gorboulev *et al*., 1997). Fig. 3 shows that the pyramidal cells in the hippocampus were stained with the antiserum and that the staining of neurons could be blocked with the antigenic peptide. The relatively poor preservation of the postmortem tissue did not allow a subcellular localization of hOCT2.

Previously, we showed that hOCT2 mediates electrogenic Na^+-independent transport of cations with diverse structures such as TEA, MPP, N^1-methylnicotinamide, and choline (Gorboulev *et al*., 1997). For rOCT1, we also demonstrated transport of monoamine neurotransmitters (Busch *et al*., 1996a). Both transporters do not translocate basic amino acids and are inhibited by various cations, including quinine and cyanine 863 (Busch *et al*., 1996b; Nagel *et al*., 1997). To evaluate the prospective neuronal functions of hOCT2 in addition to choline uptake, we investigated whether hOCT2 is capable of transporting monoamine neurotransmitters and the antiparkinsonian drugs amantadine and memantine. Transport expressed by hOCT2 was investigated by tracer influx and efflux of radioactively labeled cations and by electrical measurements with voltage-clamped *X. laevis* oocytes. Tracer uptake and efflux experiments also were performed in stably transfected human HEK 293 cells. Fig. 4 shows a tracer uptake experiment in which the cyanine 863 inhibitable uptake rates of 90 μM norepinephrine, serotonin, histamine, and dopamine and of 60 μM memantine measured with Ori buffer in the bath were compared in water-injected and hOCT2 mRNA-injected oocytes. The data show that hOCT2 is capable of translocating each of these neurotransmitters and memantine. In subsequent experiments performed in Ori buffer, the apparent K_m values of 1.9 ± 0.6 mM (norepinephrine), 1.3 ± 0.3 mM (histamine), 0.39 ± 0.16 mM (dopamine), and 0.08 ± 0.02 mM (serotonin) were obtained for the neurotransmitters. The values indicate low affinities for neu-

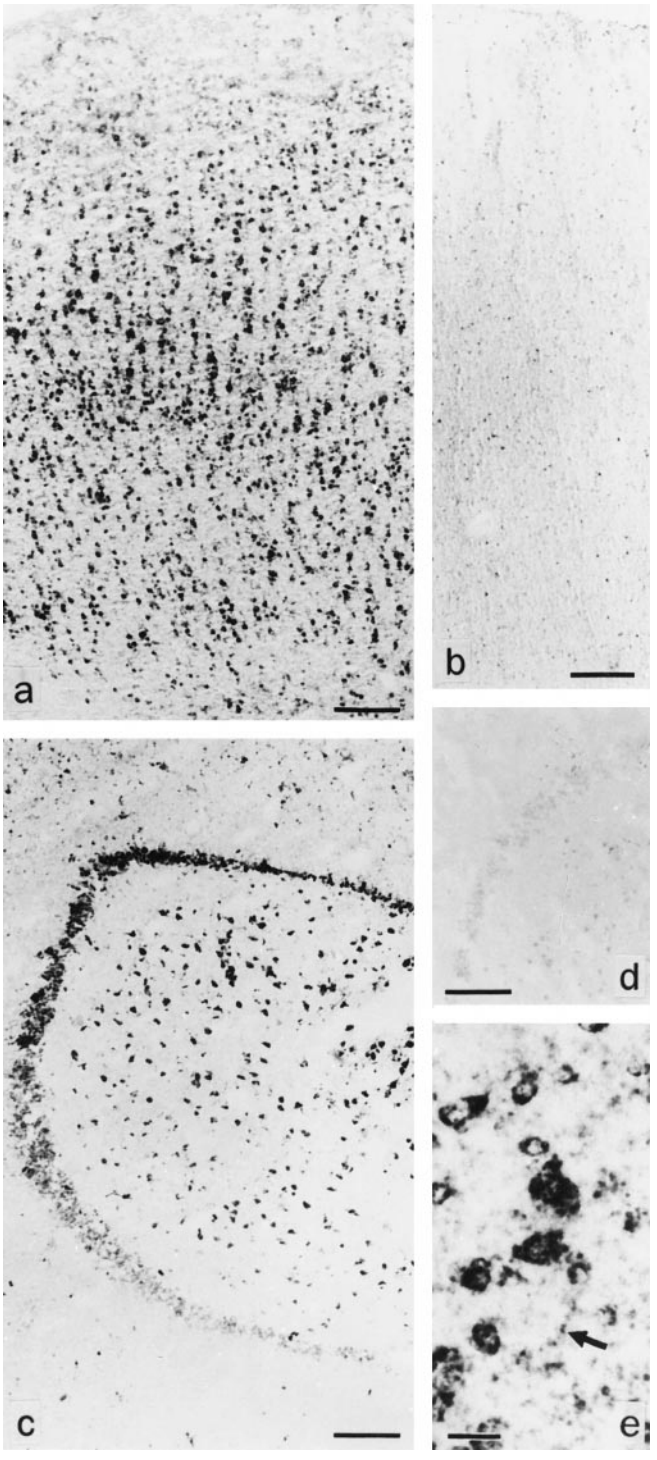

Fig. 2. *In situ* hybridization of cortex and hippocampus from the human with *hOCT2*-specific cRNA. Cryosections through area 18 of the cerebral cortex (a and b) and through the hippocampus (c, d, and e) were fixed and hybridized with an antisense (a, c, and e) and a sense (b and d) fragment of cRNA as described in Experimental Procedures. Specific hybridizations were observed in pyramidal cells of the cerebral cortex and hippocampus. *Arrow in e*, hybridization with mRNA in a dendrite. *Scale bars*: a–d 250 μm; e, 25 μm.

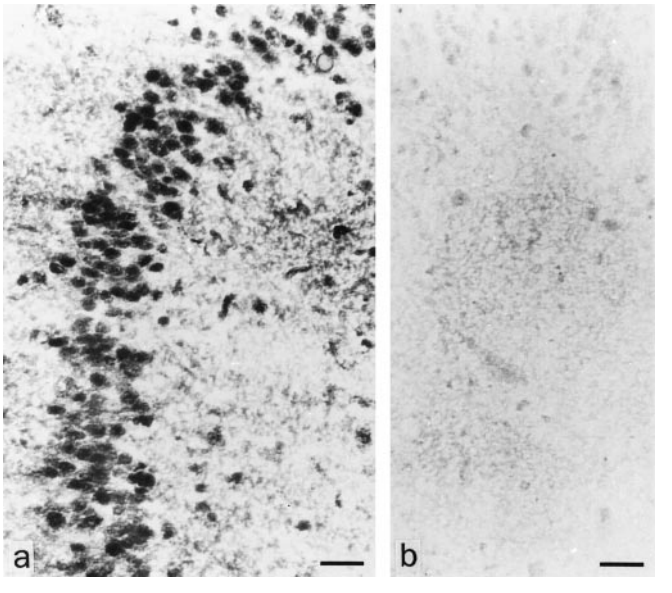

Fig. 3. Light microscopic immunohistochemical localization of hOCT2 in pyramidal cells of human hippocampus. a, Cryosections through human hippocampus were stained with a rabbit antiserum against an hOCT2-specific peptide. b, Staining of the pyramidal cells was observed, which was blocked when the antiserum was preincubated with the antigenic peptide. *Scale bars*, 50 μm.

rotransmitter transport by hOCT2. Fig. 5a shows the concentration dependence of the cyanine-sensitive dopamine uptake expressed by hOCT2. Comparing the V_{max} values of expressed neurotransmitter uptake with the uptake of choline and MPP in one batch of oocytes, the following values given in nmol/oocyte/hr were obtained: 0.42 ± 0.11 (norepinephrine), 0.30 ± 0.04 (histamine), 0.59 ± 0.13 (dopamine), 0.36 ± 0.05 (serotonin), 0.58 ± 0.13 (choline), and 0.08 ± 0.01 (MPP).

The antiparkinsonian drug amantadine has been shown to be a noncompetitive *N*-methyl-D-aspartate receptor antagonist and to increase the interstitial dopamine concentration in brain (Symchowicz *et al.*, 1973; Kornhuber *et al.*, 1995). Here, we investigated whether amantadine interacts directly with hOCT2. Fig. 5b shows high affinity inhibition of dopamine uptake in hOCT2-expressing oocytes by amantadine. An IC_{50} value of 23 ± 4 μM was determined. In Fig. 5c, we tested the capability of amantadine to *trans*-stimulate hOCT2-mediated efflux from oocytes preloaded with [^{3}H]MPP. These efflux measurements were performed as previously with rOCT1 (Busch *et al.*, 1996b). The oocytes had been stored in the presence of 1 mM choline, and the efflux measurements were performed with Ori buffer in the bath. Under these conditions, the membrane potential was ~−70 mV (see below). In water-injected control oocytes, a slow MPP efflux was observed that was identical when no cations, 0.2 mM MPP, 1.5 mM TEA, or 0.5 mM amantadine was in the bath (initial efflux rates, 0.09 ± 0.01 pmol/oocyte/hr; 12 oocytes). In hOCT2-injected oocytes, the initial efflux rate of MPP was significantly ($p < 0.01$) increased to 0.25 ± 0.03 pmol/oocyte/hr (three oocytes). This shows that hOCT2 mediates MPP efflux under *trans*-zero conditions. When 0.2 mM MPP, 1.5 mM TEA, or 0.5 mM amantadine was added to the bath, the hOCT2-mediated MPP efflux was significantly increased over that measured under *trans*-zero conditions (*trans*-MPP, 0.42 ± 0.05 pmol/oocyte/hr; *trans*-TEA, 0.51 ± 0.12 pmol/oocyte/hr; *trans*-amantadine, 0.57 ± 0.10 pmol/oocyte/hr; six oocytes; $p < 0.05$).

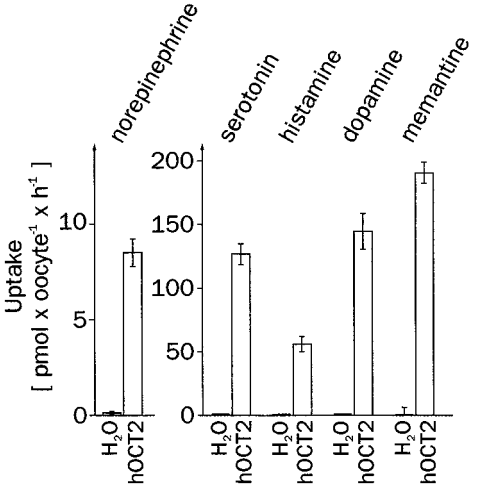

Fig. 4. Transport expression by hOCT2 in *X. laevis* oocytes of monoamine neurotransmitters and memantine. *X. laevis* oocytes were injected with 50 nl of water without or with 10 ng of hOCT2 cRNA and incubated for 3 days. Uptake of 90 μM of [^{3}H]norepinephrine, [^{3}H]serotonin, [^{3}H]histamine, or [^{3}H]dopamine and of 60 μM [^{14}C]memantine was measured in the absence or presence of the hOCT2 inhibitor cyanine 863 (36 μM). The cyanine-inhibited uptake is indicated. Median ± standard error values from 10 parallel measurements are given.

For a further characterization, electrophysiological methods were used. When hOCT2 cRNA-injected *X. laevis* oocytes were superfused with 5 mM dopamine, 50 μM memantine, or 50 μM amantadine, a reversible decrease in the membrane potential was observed, whereas water-injected control oocytes did not show a significant response (Fig. 6a). In the voltage-clamp configuration, inward currents were induced by dopamine, memantine, and amantadine (Fig. 6b) that could be inhibited by cyanine 863 (not shown). These currents showed substrate saturation and were dependent on the membrane potential (Fig. 7). With hOCT2-expressing oocytes clamped at −50 mV, half-maximal currents were induced at 0.52 ± 0.16 mM dopamine, 34 ± 5 μM memantine, and 27 ± 3 μM amantadine. These values are comparable to the apparent K_m values obtained from the uptake measure-

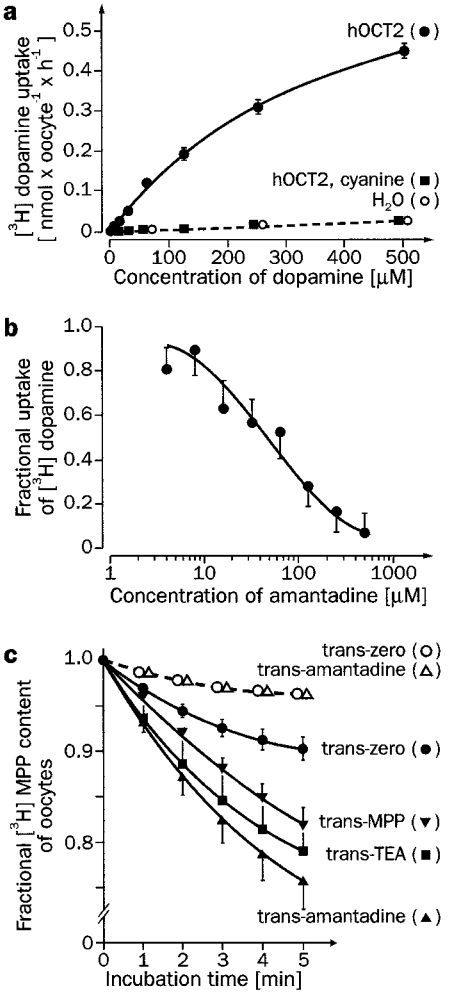

Fig. 5. Uptake and efflux of radioactively labeled cations in hOCT2-expressing oocytes of *X. laevis*. a, The substrate dependence of [^{3}H]dopamine uptake. b, Inhibition of [^{3}H]dopamine uptake by amantadine. The oocytes were injected with water (○, △) or with 10 ng/oocyte of hOCT2 cRNA (●, ▼, ■, ▲) and incubated for 3 days. a, The uptake of various concentrations of [^{3}H]dopamine was measured in the absence and presence of 75 μM cyanine 863. b, The uptake of 0.2 mM [^{3}H]dopamine was measured with hOCT2- injected oocytes in the presence of different concentrations of amantadine. c, The efflux of injected [^{3}H]MPP was measured with no cations in the bath (*trans*-zero) or with 0.2 mM MPP (*trans*-MPP), 1.5 mM TEA (*trans*-TEA), or 0.5 mM amantadine (*trans*-amantadine) in the bath. a and b, Median ± standard error values calculated from 8–10 oocytes are given. c, Mean ± standard deviation values from three oocytes are given. The lines were fitted as described in Experimental Procedures.

ments described above. With the rat organic cation transporter rOCT1, we recently observed that nontransported inhibitors like quinine and cyanine 863 may induce inward currents by inhibiting electrogenic efflux of choline (Nagel *et al*., 1997). The *trans*-inhibition of choline efflux by inhibitory cations and the electrogenic cation influx of transported cations can be distinguished because only the inward currents induced by transported cations become larger as the potential becomes more negative inside. Fig. 7b shows that the inward currents induced by 5 mM dopamine, 50 μM memantine, and 50 μM amantadine increased with increasing inside negative membrane potentials. This is consistent with the interpretation that the observed currents are caused by electrogenic translocation of dopamine, memantine, and amantadine.

Next, we investigated whether the *trans*-stimulation of MPP efflux in the experiments of Fig. 5c is due to a depolarization of the oocytes by organic cations in the bath. We performed *trans*-experiments with depolarized oocytes in which Na^+ in the bath was replaced by K^+. Electrical measurements with water-injected control oocytes showed a depolarization from -52 ± 7 to -7 ± 2 mV (>10 oocytes) after replacement of Na^+ in the bath by K^+. After preincubation of control oocytes with 1 mM choline, 0.2 mM MPP, 4 mM dopamine, or 1 mM amantadine, the membrane potential was not changed significantly. In hOCT2-expressing oocytes, membrane potentials between -40 and -50 mV were measured with Na^+ in the bath (Fig. 8). The membrane potential dropped to ~ -10 mV when Na^+ was replaced by K^+. When these oocytes were preincubated with 1 mM choline, the membrane potential was increased to -72 ± 8 mV in the presence of Na^+ and to -45 ± 14 mV in the presence of K^+ (Fig. 8). At variance, no significant increase of the membrane potential was observed after preincubation with 0.2 mM MPP, 1 mM amantadine, or 4 mM dopamine. This indicates a much smaller electrogenic efflux of these cations than choline. In Fig. 9a, we tested choline, MPP, and amantadine for *trans*-effects on MPP efflux in depolarized oocytes. This figure also shows that the efflux of [^{3}H]MPP in the presence of Na^+ was decreased when the oocytes were preincubated with choline (compare *columns 1 and 3* in Fig. 9a) and that MPP efflux was increased when Na^+ in the bath was replaced by K^+.

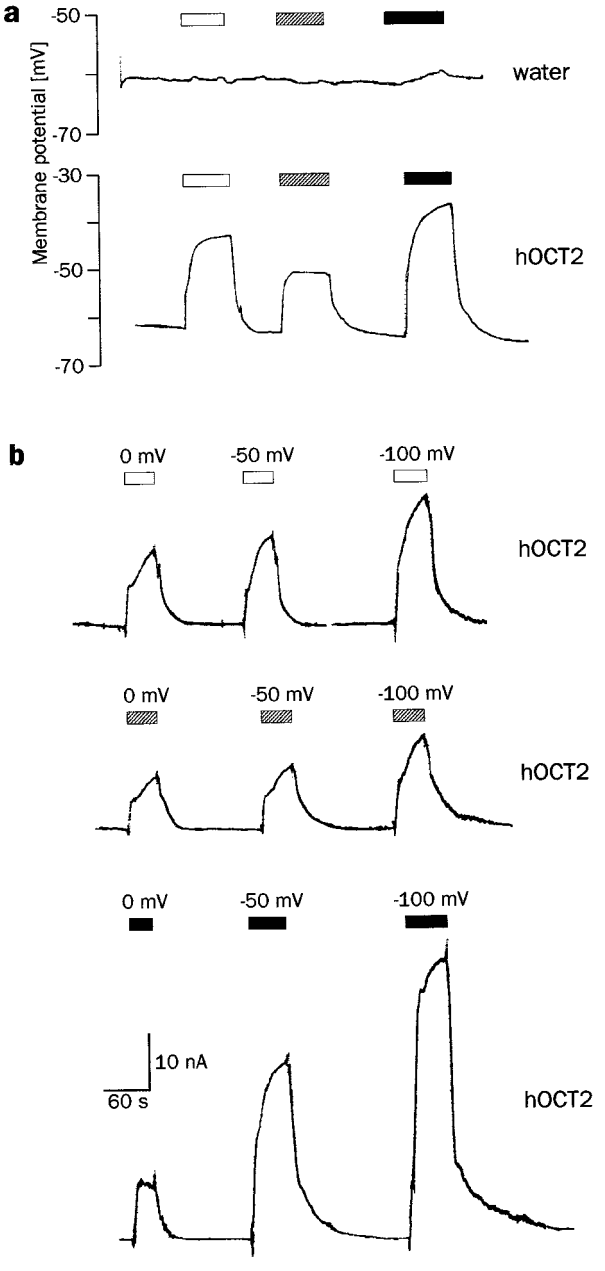

Fig. 6. Electrical measurements with *X. laevis* oocytes that were injected with *hOCT2*-cRNA or water. For expression, the oocytes were incubated for 3 days in Ori buffer. For the measurements, the oocytes with superfused with Ori buffer or with Ori containing 50 μM amantadine (□), 50 μM memantine (▨), or 5 mM dopamine (■). a, The membrane potential was measured in the absence and presence of the indicated cations. b, The oocytes were clamped to the indicated membrane potentials, and the currents were determined that were induced by superfusion of hOCT2-expressing oocytes with 50 μM amantadine, 50 μM memantine, or 5 mM dopamine.

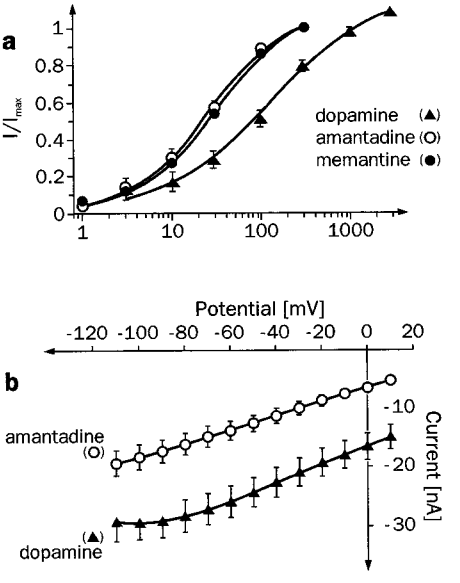

Fig. 7. Induction of currents in *hOCT2*-expressing oocytes clamped to different membrane potentials after superfusion with different concentrations of amantadine, memantine, or dopamine. a, hOCT2-cRNA-injected oocytes were clamped at -50 mV and superfused for 30 sec with the indicated cation concentrations. Mean ± standard error values of 8–10 measurements of induced currents in different oocytes are presented. The values from different oocytes were normalized against the currents induced by 1 mM TEA. b, The potential dependence of the currents induced by 5 mM dopamine or 50 μM amantadine in *hOCT2*-expressing oocytes is presented (mean ± standard error).

With depolarized oocytes, the MPP efflux was slightly *trans*-inhibited by the transported cations choline (1 mM), MPP (0.2 mM), or amantadine (1 mM) rather than being *trans*-stimulated, as in polarized oocytes (Fig. 5c). Electrical measurements showed that the addition of 1 mM choline, 0.2 mM MPP, or 1 mM amantadine to depolarized hOCT2-expressing oocytes did not lead to membrane depolarizations of >3 mV (data not shown). With the nontransported inhibitor cyanine 863 on the *trans*-side, the hOCT2-mediated MPP efflux was inhibited by ~90% (Fig. 9a). The data indicate that the *trans*-stimulation of MPP efflux observed at high membrane potentials is due to a cation-induced membrane depolarization. The *trans*-inhibition of efflux in depolarized oocytes suggests a slower out-to-in reorientation of the loaded compared with the unloaded transporter.

In Fig. 9b, we investigated *trans*-effects of organic cations on the hOCT2-mediated influx of 0.1 μM [^{3}H]MPP in depolarized oocytes. By replacement of Na^+ in the bath with K^+, the hOCT2-mediated MPP influx was reduced by ~50%. Preloading of the oocytes with 1 mM choline or 0.2 mM MPP led to a significant increase in MPP uptake. At variance, the MPP uptake was slightly reduced when the oocytes were preloaded with 1 mM amantadine or 4 mM dopamine. These data suggest different affinities or transport activities of hOCT2 for the influx and efflux of some organic cations. The *trans*-stimulation of MPP influx by choline may be partially explained by the choline-induced membrane hyperpolarization, but the *trans*-stimulation by MPP may indicate a more rapid in-to-out orientation of loaded compared with unloaded transporter forms.

In an effort to exclude possible artifacts particular to the oocyte expression system, uptake experiments were performed with HEK cells stably transfected with *hOCT2*. Fig. 10a shows the time course of MPP uptake in the *hOCT2*-transfected HEK 293 cells measured in the absence and presence of cyanine 863. In the absence of cyanine 863, a rapid initial MPP uptake was observed within 2 sec, followed by a slow further uptake that reached equilibrium within 5 min. The nonspecific uptake of MPP measured in the presence of 200 μM cyanine 863 was much slower. After a 1-hr incubation, about the same equilibrium was obtained as in the absence of cyanine 863. The effect of the membrane potential on the initial MPP uptake is investigated in Fig. 10b. After 1 sec with Na^+ in the bath, an uptake of 0.4 ± 0.1 pmol/mg of protein was determined, which was inhibited by 75 μM cyanine 863 to 0.03 ± 0.01 pmol/mg of protein (three measurements). In the presence of Na^+ (PBS buffer), membrane voltages of −44 ± 2 mV (six measurements) were determined. After replacement of Na^+ by K^+ (K buffer), the membrane potentials were +6 ± 1 mV (six measurements). With K^+ in the bath, the uptake after 1 sec was reduced to 0.14 ± 0.04 pmol/mg of protein (three measurements). This was expected for potential-dependent transport. The concentration dependence of the cyanine-sensitive uptake of MPP

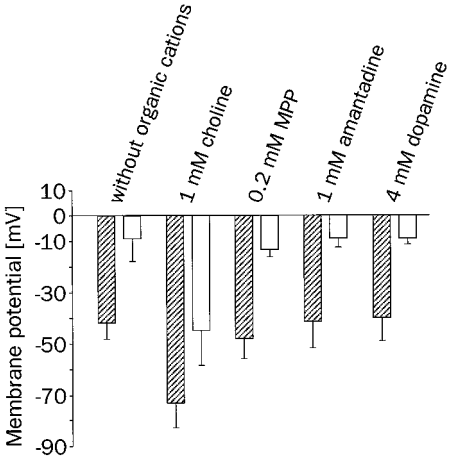

Fig. 8. Membrane potentials of *hOCT2*-expressing oocytes without and with preincubation with organic cations that were measured with Na^+ or K^+ in the bath. hOCT2-cRNA-injected oocytes were preincubated for 12 hr without organic cations or with the indicated concentrations of choline, MPP, amantadine, and dopamine. The membrane potential was measured with Ori buffer in the bath (▨) or after replacement of Na^+ in the Ori buffer by K^+ (□). Mean ± standard deviation values from six oocytes are shown.

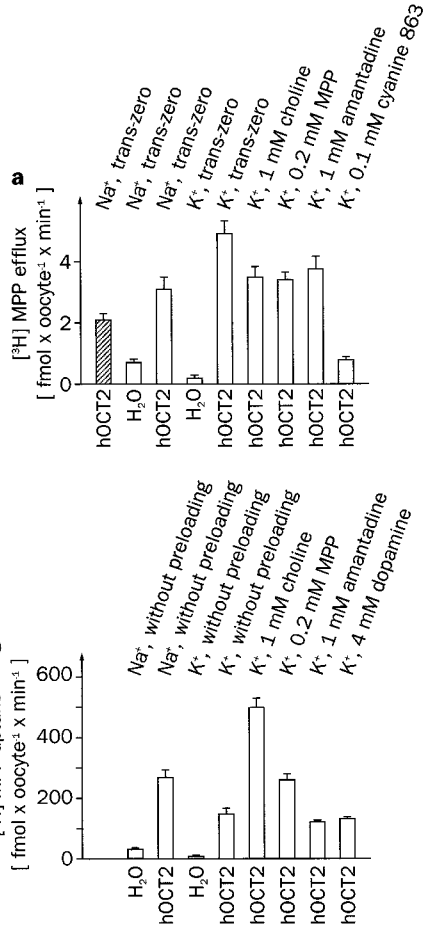

Fig. 9. The *trans*-effects of organic cations on MPP efflux (a) and MPP influx (b) in depolarized oocytes. Water-injected control oocytes or oocytes expressing *hOCT2* were injected with 0.1 pmol of [^{3}H]MPP (efflux) or with different amounts of nonradioactive cations (preloading for uptake measurements). The indicated intracellular cation concentrations were estimated from the injected amounts of cations by assuming an intracellular aqueous space of 0.5 μl. a, Initial [^{3}H]MPP efflux rates were estimated by measuring the efflux of washed oocytes between 10- and 70-sec incubations in Ori buffer (Na^+, *trans*-zero) or in K oocyte buffer without or with the indicated cations (depolarized oocytes: K^+ *trans*-zero or K^+ cations). ▨, *trans*-zero condition in Fig. 5c in which the oocytes had been stored in the presence of 1 mM choline. b, The initial MPP uptake rates were estimated from the uptake after a 5-min incubation in Ori buffer (Na^+ without preloading) or in K oocyte buffer (K^+ without preloading or K^+ cation concentration) containing 0.1 μM [^{3}H]MPP. Mean ± standard error values from 8–10 oocytes are presented.

and dopamine in *hOCT2*-transfected and in nontransfected HEK 293 cells is shown in Fig. 11. Only in the transfected cells was significant cyanine-inhibitable cation uptake observed. The apparent K_m values for hOCT2-mediated influx of MPP (16 ± 3 μM) and dopamine (0.33 ± 0.13 mM) were not significantly different from the values obtained after expression of hOCT2 in oocytes (see above and Gorboulev *et al.*, 1997). Because the expression of cation transport by hOCT2 in stably transfected HEK 293 cells varied during the cultivation, the V_{max} values of MPP, choline, and dopamine were compared within one batch of cells. In such an experiment, V_{max} values of 2.7 ± 0.4 (MPP), 16.8 ± 1.5 (choline), and 19.4 ± 3.0 (dopamine) nmol/mg of protein/min were determined. As already observed with oocytes, similar V_{max} values were obtained for dopamine and choline, whereas the V_{max} value for MPP was much smaller.

To further investigate *hOCT2* for symmetry, we compared the influx and efflux rates of choline and MPP in depolarized HEK 293 cells at their respective K_m concentrations (Gorboulev *et al.*, 1997). Again, the depolarization was achieved by raising the extracellular K^+ concentration. Fig. 12 shows efflux of 19 μM [^{3}H]MPP and 210 μM [^{3}H]choline in *hOCT2*-transfected and in nontransfected HEK 293 cells measured in the presence of Na^+ or K^+. In the control cells, small initial efflux rates were determined. In the *hOCT2*-transfected HEK 293 cells, the efflux of choline and MPP was significantly higher and was increased after depolarization of the cells (shown for MPP). In the depolarized cells, initial efflux rates of 2.3 ± 0.2 and 5.2 ± 1.3 pmol/mg of protein/sec were estimated for 19 μM [^{3}H]MPP and 210 μM [^{3}H]choline, respectively. For uptake of 19 μM [^{3}H]MPP and 210 μM [^{3}H]choline in depolarized cells, initial uptake rates of 12 ± 2 and 54 ± 11 pmol/mg of protein/sec were determined. The data show ~5-fold higher uptake rates for the influx of these cations than for their efflux. This may indicate an asymmetry of the transporter in the depolarized membrane. In Fig. 13, we compare *trans*-effects of organic cations on the influx and efflux of 0.1 μM [^{3}H]MPP in depolarized HEK 293 cells expressing hOCT2. The results are consistent with those in the oocytes. The MPP efflux in depolarized *hOCT2*-transfected HEK 293 cells was *trans*-inhibited by 10 mM choline, 1 mM MPP, and 1 mM amantadine, but the uptake of MPP was *trans*-stimulated by 10 mM choline and 1 mM MPP but slightly *trans*-inhibited by 1 mM amantadine and 4 mM dopamine. The data indicate an asymmetry of hOCT2 for transport of choline and MPP.

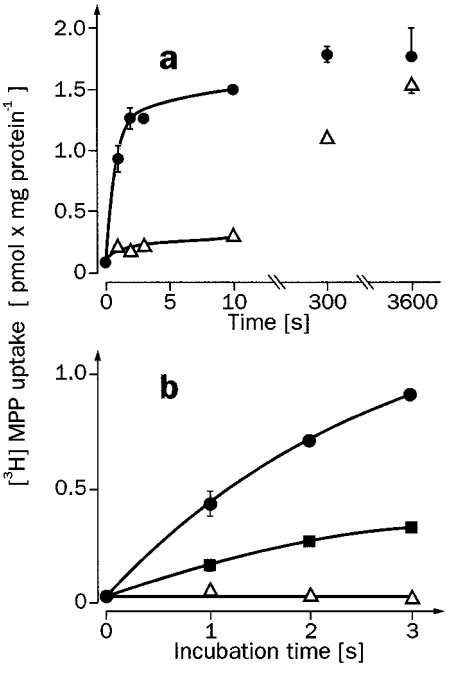

Fig. 10. Time course and potential dependence of hOCT2-mediated uptake of MPP in HEK 293 cells. HEK 293 cells that were constantly transfected with *hOCT2* were incubated with 0.1 μM [^{3}H]MPP, and the cellular MPP concentration was determined after different time intervals. The measurements were performed in the absence (●, ■) or presence (△) of 200 μM cyanine 863 with either Na^+ (●, △) or K^+ (■) in the bath. Mean ± standard error values of three measurements are presented.

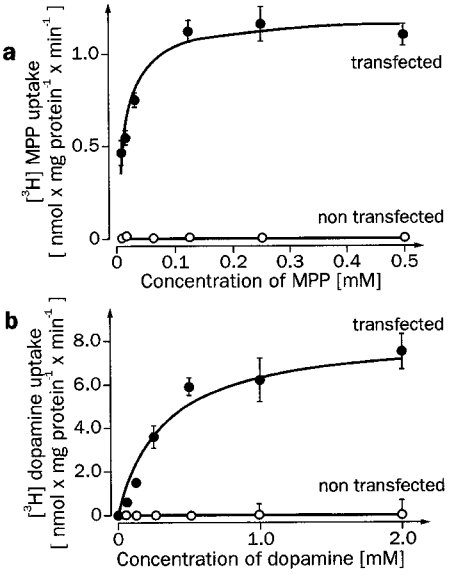

Fig. 11. Concentration dependence of hOCT2-mediated uptake of [^{3}H]MPP and [^{3}H] dopamine in HEK 293 cells. Initial uptake rates at different concentrations of MPP or dopamine were determined after 1-sec incubation of nontransfected HEK 293 cells or HEK 293 cells that were constantly transfected with *hOCT2*. The measurements were performed in the presence of Na^+ without or with 200 μM cyanine 863 in the bath. Cyanine-inhibited uptake rates are presented that were calculated from three to six parallel measurements. Mean ± standard error values are indicated. The curves were obtained by fitting the Michaelis-Menten equation to the data.

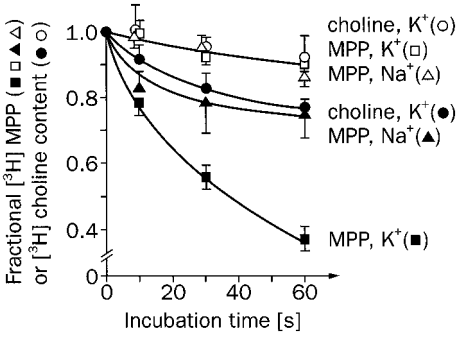

Fig. 12. Efflux of MPP and choline from *hOCT2*-transfected HEK 293 cells measured under *trans*-zero conditions. Nontransfected HEK 293 cells (○, □, △) and HEK 293 cells stably transfected with *hOCT2* (●, ▲, ■) were preloaded with 19 μM [^{3}H]MPP (□, ■, △, ▲) or 210 μM [^{3}H]choline (○, ●). After washing at 0°, the efflux of the radioactively labeled cations was measured at 37° under *trans*-zero conditions with Na^+ or K^+ in the bath. Mean ± standard deviation values of four determinations are presented.

To evaluate the possible relevance of hOCT2 regarding the therapeutic actions of amantadine in brain during treatment of Parkinson's disease, we tested whether amantadine also affects the hDAT that mediates high affinity uptake of dopamine into dopaminergic neurons (Giros *et al.*, 1992; Sonders *et al.*, 1997). To examine the action by amantadine on transport by hDAT, *X. laevis* oocytes expressing hDAT were superfused with 10 μM dopamine, 50 μM amantadine, or 10 μM dopamine plus 50 μM amantadine. Drug-elicited currents were measured in voltage-clamped oocytes during a series of jumps to a range of membrane potentials (Fig. 14). Amantadine itself did not elicit transport-associated current at hDAT, and in combination with dopamine, it showed no significant inhibition of the transport-associated current of dopamine. These data indicate that at a concentration equivalent to its apparent K_m value for hOCT2, amantadine has no discernible effect on hDAT.

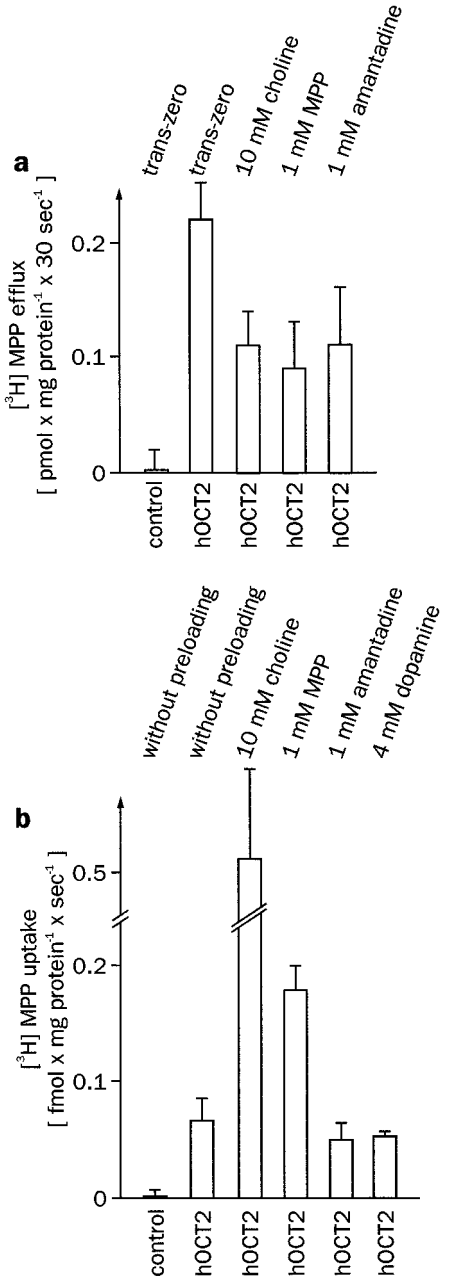

Fig. 13. The *trans*-effects of organic cations on the efflux (a) and influx (b) of MPP in depolarized HEK 293 cells stably transfected with *hOCT2*. a, *hOCT2*-transfected HEK 293 cells and nontransfected control cells were preloaded with 0.1 μM [^{3}H]MPP and washed at 0°, and the [^{3}H]MPP efflux was measured after a 30-sec incubation at 37° in the K buffer. b, The transfected and nontransfected cells were preincubated without and with the indicated organic cations and washed at 0° with K buffer. The cells were incubated for 1 sec at 37° in K buffer containing 0.1 μM [^{3}H]MPP, and the cellular [^{3}H]MPP was analyzed. Mean ± standard deviation values from four determinations are presented.

Discussion

hOCT2 is a polyspecific electrogenic cation transporter originally shown to be expressed in kidney that translocates small organic cations with diverse structures, including choline and MPP (Gorboulev *et al.*, 1997). We report that hOCT2 also is expressed in neurons of various brain areas, including cerebral cortex, hippocampus, corpus striatum, nucleus amygdaloideus, and thalamus. hOCT2 is not identical to the Na^+-independent corticosterone-sensitive extraneuronal norepinephrine uptake$_2$ transporter from myocardial cells (Iversen, 1967; Trendelenburg, 1988), which also is expressed in the human glioma cell line SK-MG-1 (Streich *et al.*, 1996). hOCT2 has a >100-fold lower sensitivity to corticosterone (data not shown) and is not transcribed in SK-MG-1 cells. Evidence is presented that hOCT2 also mediates low affinity transport of the monoamine neurotransmitters dopamine, norepinephrine, serotonin, and histamine. In distinction to the Na^+/Cl^--dependent high affinity transporters for norepinephrine, dopamine, and serotonin whose expression is largely restricted to the neurons that synthesize these neurotransmitters, expression of hOCT2 is relatively widespread across regions of the human brain. In light of the broad substrate specificity of hOCT2, this polyspecific cation transporter may serve the function in brain of limiting the action of aminergic neurotransmitters that have evaded high affinity uptake mechanisms. Mental or psychiatric alterations or diseases such as depression and schizophrenia have been linked to disturbances in dopamine transport, but there are no links to the high affinity Na^+/Cl^--dependent dopamine transporters (Seeman and Niznik, 1990; Gelernter *et al.*, 1995; Maier *et al.*, 1996). It therefore will be interesting to analyze the putative role of hOCT2 in such disorders.

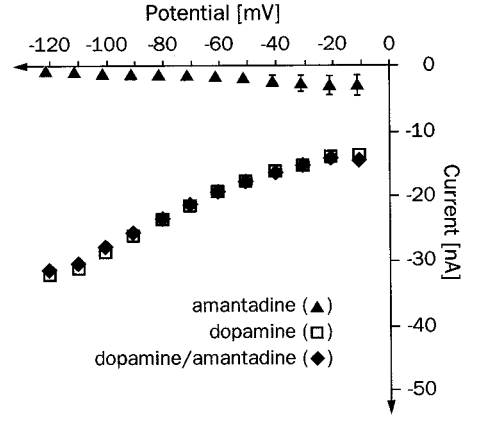

Fig. 14. hDAT-mediated currents were measured in Ori buffer at different membrane potentials, which were induced by superfusion with dopamine and amantadine. Superfusion of hDAT-expressing oocytes was performed with 10 μM dopamine, 50 μM amantadine, or 10 μM dopamine plus 50 μM amantadine.

hOCT2 transports the neurotoxin MPP with a similar affinity as hDAT (Giros *et al*., 1992). MPP is an cerebral oxidation product of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrine that passes the blood-brain barrier. In humans and in several animal models, an injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrine causes a selective degeneration of dopaminergic neurons that project from the substantia nigra to the corpus striatum, which leads to Parkinson′s disease (Chiueh *et al*., 1985; Snyder and D′Amato, 1986; Kinemuchi *et al*., 1987; Tipton and Singer, 1993). The selective degeneration of these neurons by MPP probably is due to MPP uptake via hDAT in the nerve terminals or to other factors as the intracellular binding to neuromelanin rather than to MPP uptake via hOCT2. However, hOCT2 may be involved indirectly because neuronal uptake by this transporter will reduce the overall concentration of MPP or of environmental MPP-like toxins that may induce Parkinson's disease (Calne and Langston, 1983). Recently, an autosomal recessive form of juvenile parkinsonism was localized to a fragment of chromosome 6q25.2–27 (Matsumine *et al*., 1997). We have localized the gene of hOCT2 to chromosome 6q26 (Koehler *et al*., 1997), which represents an intriguing proximity to the gene defect of juvenile parkinsonism.

An intriguing finding was that the antiparkinsonian drugs amantadine and memantine are transport substrates and competitive inhibitors of hOCT2. It is noteworthy that the affinity of amantadine to hOCT2 is in the same range as the amantadine concentrations in the serum or cerebrospinal fluid (4–17 μM) that were effective for the symptomatic treatment of Parkinson′s disease (Kornhuber *et al*., 1995). Although the pharmacological action of amantadine may also involve noncompetitive *N*-methyl-D-aspartate receptor antagonism, muscarinic mechanisms, and dopamine release from neuronal storage sites (Grelak *et al*., 1970; Parkes, 1974; Kornhuber *et al*., 1991; Chen *et al*., 1992), the current data provide the first evidence that this compound may increase extracellular neurotransmitter concentration by inhibition of dopamine uptake via hOCT2. No other neurotransmitter transporter has been identified as a target for these compounds; moreover, we report that the high affinity dopamine transporter hDAT is insensitive to therapeutically relevant concentrations of amantadine, as suggested previously (Sonders *et al*., 1997). hOCT2-mediated uptake of amantadine into neurons also could explain the accumulation of amantadine in brain observed during the first days of treatment (Kornhuber *et al*., 1995).

Here, we further characterize transport properties of hOCT2. In measurement of the *trans*-effects in depolarized oocytes and HEK 293 cells, a functional asymmetry of hOCT2 mediated influx and efflux was observed for some cations, whereas the transporter may operate symmetrically for others. In depolarized cells, choline, MPP, dopamine, and amantadine slightly inhibited MPP efflux. At variance, the influx of MPP was *trans*-inhibited by dopamine and amantadine and *trans*-stimulated by choline and MPP. The data suggest that the in-to-out reorientation of transporter forms is accelerated after loading with MPP or choline from the inside but remains unchanged or is slightly decreased after loading with dopamine or amantadine. The out-to-in orientation of transporter forms may be generally slowed down after loading with cations from the outside. This suggests different cation specificities for cation influx and efflux. Combining tracer flux and electrical measurements, we showed that hOCT2-mediated cation influx and efflux rates in polarized cells are significantly determined by the membrane potential. For example, it could be demonstrated that the *trans*-stimulation of cation efflux in polarized cells measured for rOCT1 (Busch *et al*., 1996b) and hOCT2 (current report) is a consequence of the membrane depolarization by transported cations on the outside.

The detailed physiological role of hOCT2 for the cerebral homeostasis of choline, cationic drugs, and xenobiotics in relation to Na^+-dependent choline and monoamine transporters can only be determined by measurements with human brain slices or synaptosomes. This is a demanding task because well preserved shock-frozen human brain tissue is required, and it may be difficult to quantify the activities of different transporters in different cell types. Currently, the reported localization and functional characteristics of hOCT2 suggest that this transporter affects interstitial and neuronal concentrations of organic cations in brain. The observed *trans*-effects of intracellular choline on cation uptake indicate that the capacity of hOCT2 to translocate cationic drugs and xenobiotics from brain interstitium can be modulated by the intraneuronal choline concentrations. In summary, our data demonstrate the expression of hOCT2 in human brain and suggest that it could represent a "background transporter" for monoamine neurotransmitters. hOCT2 inhibition by the antiparkinsonian drugs amantadine and memantine, at clinically relevant concentrations, provides the first evidence for the mechanism by which these compounds increase extracellular dopamine concentrations.

Acknowledgments

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