Identity of the Organic Cation Transporter OCT3 as the Extraneuronal Monoamine Transporter (uptake2) and Evidence for the Expression of the Transporter in the Brain*

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We investigated the transport of cationic neurotoxins and neurotransmitters by the potential-sensitive organic transporter OCT3 and its steroid sensitivity using heterologous expression systems and also analyzed the expression of OCT3 in the brain. When expressed in mammalian cells, OCT3 mediates the uptake of the neurotoxin 1-methyl-4-phenylpyridinium (MPP⁺) and the neurotransmitter dopamine. Competition experiments show that several cationic neuroactive agents including amphetamines interact with OCT3. When expressed in Xenopus laevis oocytes, OCT3-mediated MPP⁺ uptake is associated with inward currents under voltage-clamp conditions. The MPP⁺-induced currents are saturable with respect to MPP⁺ concentration, and half-maximal saturation (Kₐ,½) occurs at about 25 μM MPP⁺ with membrane potential clamped at −50 mV. The Kₐ,½ for MPP⁺ is markedly influenced by membrane potential. OCT3 is inhibited by several steroids, and β-estradiol is the most potent inhibitor (Kᵢ ≈ 1 μM). The pattern of steroid sensitivity of OCT3 is different from that of OCT1 and OCT2 but correlates significantly with that of the extraneuronal monoamine transporter (uptake2). The transport characteristics and steroid sensitivity provide strong evidence for the molecular identity of OCT3 as uptake2. OCT3 is expressed in the brain as evidenced from Northern blot analysis, reverse transcription-polymerase chain reaction, and in situ hybridization using OCT3-specific probes. The molecular identity of the transcript hybridizing to the probe has been established by sequencing the reverse transcription-polymerase chain reaction product and also by the isolation of the OCT3 cDNA from a brain cDNA library. Regional distribution studies with in situ hybridization show that OCT3 is expressed widely in different brain regions, especially in the hippocampus, cerebellum, and cerebral cortex. OCT3 is likely to play a significant role in the disposition of cationic neurotoxins and neurotransmitters in the brain.
the cationic neurotransmitters 1-methyl-4-phenylpyridinium (MPP+) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine are likely to be substrates for this transporter.

Several studies have provided evidence for the existence of a catecholamine transport system that is different from the transporters present in monoaminergic neurons (19–21). This transporter is referred to as the extraneuronal monoamine transporter or uptake2. The distinguishing characteristics of uptake2 include the ability to transport catecholamines in a Na+- and Cl−-independent manner, interaction with a variety of organic cations, dependence on the membrane potential as the driving force, inhibition by steroids, and expression in a wide variety of tissues. The uptake2 system is of physiological and pharmacological relevance to the metabolism of catecholamines (19–21). The molecular identity of this transport system has not yet been established. The functional characteristics of uptake2 suggest that it may be a potential-sensitive organic cation transporter, but OCT1 and OCT2 are excluded as potential candidates based on tissue distribution (14, 17). The possibility that OCT3 may be identical to uptake2 has not yet been examined.

Therefore, we have undertaken the current investigation to characterize in detail the transport of cationic neurotransmitters and neurotransmitters by OCT3 and its sensitivity to steroids. The characterization of OCT3 transport function was carried out within the present study by using two different heterologous expression approaches. A mammalian cell expression system in which the transport function of OCT3 was monitored by the uptake of radiolabeled MPP+ or TEA and the Xenopus laevis oocyte expression system in which the transport function of OCT3 was monitored by electrophysiological means using unlabeled MPP+ as the substrate. The results of these studies show that OCT3 interacts with a wide variety of cationic neurotransmitters and neurotransmitters and also with steroids. The transport characteristics and steroid sensitivity of OCT3 provide strong evidence for its molecular identity as the extraneuronal monoamine transporter (uptake2). We also provide in this report unequivocal evidence for the expression and region-specific distribution of this transporter in the brain. These studies raise the possibility that OCT3 may play a significant role in the handling of cationic neurotransmitters and neurotransmitters in the brain.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]MPP+ (specific radioactivity, 83 Ci/mmol) and [ethyl-1-14C]tetraethylammonium bromide (specific radioactivity, 55 mCi/mmol) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [3H]Dopamine (specific radioactivity, 20.3 Ci/mmol) was obtained from NEN Life Sciences Products. Unlabeled MPP+, amphetamines, dopamine and other organic cations were obtained from Research Biochemicals International (Natick, MA) or Sigma. Steroids were obtained from Sigma. Cell culture media and Lipofectin were from Life Technologies, Inc. Restriction enzymes were from Promega (Madison, WI). Magna nylon transfer membranes were purchased from Micro Separations, Inc. (Western, MA). HEK cells were obtained from the American Type Culture Collection (Manassas, VA). Human retinal pigment epithelial cells (HRPE) were originally provided by Dr. M. A. Del Monte (University of Michigan, Ann Arbor, MI) and have been in use in our laboratory for several years (22, 23). The RNA transcription kit mMESSAGE mMACHiNE™ for synthesis of cRNA was obtained from Ambion (Austin, TX). The Trizol reagent for the isolation of total RNA and oligo(dT)-cellulose for purification of poly(A) RNA were from Life Technologies, Inc., and the ready-to-go oligolabeling kit used in the preparation of cDNA probes was from Amersham Pharmacia Biotech. X. laevis were purchased from Nasco (Fort Atkinson, WI).

**Functional Expression of OCT cDNAs in HRPE Cells**—The rat OCT3 cDNA was originally cloned from a rat placental CDNA library (18). A full-length rat OCT2 cDNA was isolated by screening a rat liver cell line cDNA library with rat OCT3 cDNA as a probe. The clone was sequenced and confirmed of its identity. The sequence of the clone was identical to the sequence of rat kidney OCT1 cDNA reported previously by Grunemann et al. (8). A full-length rat OCT2 cDNA was obtained by RT-PCR using rat OCT2-specific primers and rat kidney poly(A)+ RNA. The resultant product was subcloned into pGEM-T vector, and the identity of the insert was confirmed by sequencing. The cDNA contained the complete coding region and corresponded to nucleotide positions 20–4200 of the rat OCT2 cDNA and the rat OCT2 cDNA (23). The cloned OCT1, OCT2, and OCT3 cDNAs were oriented in the plasmid in such a way that their expression was under the control of the T7 promoter. The cDNAs were heterologously expressed in HRPE or HeLa cells by vaccinia virus expression system as described previously (24, 25). Transport measurements were made at room temperature using [3H]dopamine (H3Dopa; [3H]TEA. The transport buffer was composed of 25 mM Tris, Heps (pH 8.5), supplemented with 280 mM mannotil, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, and 5 mM glucose. When the influence of pH on transport was investigated, transport buffers of different pH were prepared by varying the concentrations of Tris, Heps, and Mes. In some experiments, mannitol in the transport buffer was isosmotically substituted with N-methyl-d-glucamine chloride, sodium gluconate, or NaCl to assess the role of Na+ and Cl− in the transport process. In experiments dealing with steroid inhibition, the transport buffer contained 1% dimethyl sulfoxide to keep the steroids in solution. After incubation for a desired time, transport was terminated by aspiration of the uptake buffer followed by two washes with 2 mM of ice-cold transport buffer. After this, the cells were solubilized with 0.5% SDS and transferred to vials for quantitation of the radioactivity associated with the cells. HRPE or HeLa cells transfected with empty vector under similar conditions served as control. In experiments dealing with saturation kinetics, data were analyzed by nonlinear regression and confirmed by linear regression. IC50 (concentration at which the inhibition was 50%) were calculated from the dose-response data for the inhibition of [3H]MPP+ or [14C]TEA uptake by various cationic neurotoxins/neurotransmitters or steroids.

**cRNA Synthesis**—The pSPORT-OCT3 cDNA construct was linearized with NotI, and the cDNA insert was transcribed in vitro using T7 RNA polymerase in the presence of ribonucleoside inhibitor and RNA cap analog. The mMESSAGE mMACHiNE™ kit was employed for this purpose.

**Northern Blot Analysis**—Total RNA isolated from different tissues from rat was subjected to oligo(dT)-cellulose chromatography to purify poly(A)+ mRNA. mRNA samples (5 μg/lane) were size-fractionated and probed sequentially with rOCT1, rOCT2, and rOCT3 cDNAs under high stringency conditions. The cDNA probes were radiolabeled with [α-32P]dCTP by random priming using the ready-to-go oligolabeling kit.

**RT-PCR and Restriction Analysis**—Poly(A)+ mRNA samples from rat brain and rat placenta were used for RT-PCR. The rOCT3-specific primers used in the analysis were 5′-CCA TCG TCA GCC AGT TT-3′ (upstream) and 5′-ACA CGA CAC CCC TGC CAC TA-3′ (downstream). These primers correspond to the nucleotide positions 789–808 and 1620–1639 in the full-length rOCT3 cDNA (18). The expected size of the RT-PCR product, predicted from the positions of the primers, is 500 bp. The resulting RT-PCR products were gel-purified and used for DNA sequencing analysis. The sequencing primers were used to sequence DNA products for PuII, SacI, and TaqI. The expected sizes of the digestion products were 385 and 466 bp for PuII, 770 and 81 bp for SacI, and 294 and 557 bp for TaqI.

**Screening of the CDNA Library**—The CDNA library used for screening was a 1.4-kbp fragment of rat OCT3 cDNA obtained by digestion with SacI/NotI. The cDNA probe was labeled with [α-32P]dCTP using the
RESULTS

OCT3-mediated MPP⁺ Uptake in HeLa Cells Versus HRPE Cells—HeLa cells are widely used for heterologous expression of a variety of cloned transporters. Analysis of the function of cloned transporters is facilitated if the cells used in heterologous expression possess little or no endogenous transport activity that could be ascribed to the cloned transporter. This eliminates the need to differentiate between the endogenous activity and the cDNA-mediated activity in cells expressing heterologously the cloned transporter. We have shown previously that transfection of HeLa cells with rat OCT3 cDNA led to a severalfold increase in the uptake of the organic cation TEA (18). However, under the same conditions, the increase in the uptake of the organic cation guanidine was much smaller. This was primarily because of the presence of relatively high endogenous guanidine uptake in HeLa cells compared with TEA uptake. Because the purpose of the present investigation was to characterize the OCT3-mediated transport of the cationic neurotoxin MPP⁺, it was necessary first to assess the suitability of HeLa cells for this purpose by determining the relative contribution of endogenous MPP⁺ uptake activity to the total MPP⁺ uptake measured in OCT3 cDNA-transfected cells (Fig. 1A). OCT3 cDNA did induce MPP⁺ uptake in these cells, but the increase was small (∼50%). Thus, the endogenous activity was found to contribute 60–70% to the activity measured in cDNA-transfected cells. Furthermore, the endogenous MPP⁺ uptake was saturable as evidenced from the inhibition of [³H]MPP⁺ uptake by unlabeled MPP⁺. Interestingly, the potency of unlabeled MPP⁺ to inhibit the uptake of [³H]MPP⁺ was markedly different between control cells and OCT3-expressing cells. These data indicate that the endogenous MPP⁺ uptake activity in HeLa cells is not mediated by constitutively expressed OCT3. However, the presence of relatively high endogenous MPP⁺ uptake activity made HeLa cells less suitable for detailed characterization of OCT3-mediated MPP⁺ uptake.

We then used a human retinal pigment epithelial cell line (HRPE) for heterologous expression of OCT3. In contrast to the results from HeLa cells, transfection of HRPE cells with OCT3 cDNA led to a 3-fold increase in MPP⁺ uptake (Fig. 1B). In addition, the endogenous MPP⁺ uptake activity was not saturable with respect to MPP⁺ because unlabeled MPP⁺ failed to inhibit [³H]MPP⁺ uptake in control cells. The uptake in cDNA-transfected cells was however saturable. When the cDNA-specific [³H]MPP⁺ uptake was considered, unlabeled MPP⁺ inhibited OCT3-mediated [³H]MPP⁺ uptake with an IC₅₀ value of 138 ± 11 μM. These results show that HRPE cells are more suitable than HeLa cells for the investigation of OCT3-mediated MPP⁺ uptake. Subsequent experiments were therefore carried out with HRPE cells.

pH Dependence and Saturation Kinetics of OCT3-mediated MPP⁺ Uptake in HRPE Cells—The influence of extracellular pH on the uptake of MPP⁺ was then evaluated in control cells and in OCT3-expressing cells. The MPP⁺ uptake activity in both groups of cells was influenced by extracellular pH. The activity increased dramatically when the pH of the extracellular medium was changed from 5.5 to 8.5. The endogenous MPP⁺ uptake activity increased 2.5-fold when the pH of the extracellular medium was changed from 5.5 to 8.5 (data not shown). When OCT3-specific uptake was alone considered, the uptake increased almost 15-fold at pH 8.5 compared with pH 5.5 (Fig. 2A). The OCT3-specific uptake measured at pH 8.5 was saturable, mediated by a single transport system with an apparent Michaelis-Menten constant (Kₘ) of 91 ± 10 μM and a maximal velocity (Vₘₐₓ) of 5.3 ± 0.3 nmol/10⁶ cells/30 min (Fig. 2B). In both groups of cells, the activity of OCT3-mediated uptake increased about 3-fold when the pH was increased from 5.5 to 7.0 (data not shown), whereas the activity of endogenous uptake was decreased at higher pH (5.5–8.5) (Fig. 3A). The pH-dependence was similar for both groups of cells (Fig. 3B). The pH optimum for OCT3-mediated uptake was at pH 7.0, whereas the pH optimum for endogenous uptake was at pH 5.5 (Fig. 3C).
The influence of the cationic compounds on the endogenous uptake in OCT3-expressing cells. We were thus able to compare subtracting the uptake in control cells from the corresponding uptake measured in cDNA-transfected cells. The OCT3-specific uptake was calculated by subtracting the uptake in cDNA-transfected cells from the uptake in vector-transfected cells. When OCT3-specific uptake alone was considered, all six compounds (range, 1 μM-10 mM) had little or no effect on [3H]MPP⁺ uptake. The order of inhibitory potency was amphetamine > serotonin (Fig. 3B). ND, not determinable.

| Inhibitor   | Endogenous uptake IC₅₀ (mM) | OCT3-specific uptake IC₅₀ (mM) |
|-------------|-----------------------------|-------------------------------|
| Amphetamine | 0.060 ± 0.008               | 0.042 ± 0.007                 |
| Desipramine | 0.013 ± 0.004               | 0.068 ± 0.002                 |
| MPP⁺        | ND                          | 0.143 ± 0.010                 |
| Methamphetamine | 0.128 ± 0.025          | 0.247 ± 0.026                 |
| Dopamine    | 11.3 ± 2.0                  | 0.62 ± 0.04                   |
| Serotonin   | 4.5 ± 1.7                   | 0.97 ± 0.18                   |
OCT3 Expression in the Brain

TABLE II

Inhibition of OCT3-mediated MPP⁺ uptake by various organic cations

HRPE cells were transfected with either pSPORT-OCT3 cDNA or pSPORT alone. Uptake of [³H]MPP⁺ (1 μM) was measured in these cells in mannitol medium (pH 8.5) with a 30-min incubation. Uptake was measured in the absence or presence of 5 mM organic cations (pH of the medium was adjusted to 8.5 in each case). Values in parentheses are the percent of corresponding control uptake. TMA, tetramethylammonium; NMN, N¹,methylnicotinamide.

| Inhibitor         | pSPORT [³H]MPP⁺ Uptake (pmol/10⁶ cells/30 min) | pSPORT-OCT3 cDNA [³H]MPP⁺ Uptake (pmol/10⁶ cells/30 min) | cDNA-specific [³H]MPP⁺ Uptake (pmol/10⁶ cells/30 min) |
|-------------------|-----------------------------------------------|--------------------------------------------------------|------------------------------------------------------|
| None              | 17.2 ± 1.3 (100)                              | 35.7 ± 2.3 (100)                                        | 37.5 (100)                                            |
| Dimethylamiloride | 2.1 ± 0.1 (15)                                | 1.9 ± 0.1 (15)                                          | -0.2 (-1)                                             |
| Clonidine         | 3.4 ± 0.1 (18)                                | 11.2 ± 0.2 (19)                                         | 7.8 (19)                                              |
| TEA               | 10.8 ± 0.2 (75)                               | 18.5 ± 0.2 (38)                                         | 8.0 (23)                                              |
| Guanidine         | 15.9 ± 0.6 (84)                               | 27.9 ± 0.4 (47)                                         | 12.0 (25)                                             |
| Dimethylamiloride | 16.7 ± 0.5 (88)                               | 31.8 ± 0.4 (64)                                         | 15.1 (37)                                             |
| Clonidine         | 16.1 ± 0.2 (85)                               | 34.3 ± 0.7 (58)                                         | 18.2 (45)                                             |
| TMA               | 17.3 ± 0.4 (91)                               | 37.8 ± 1.1 (64)                                         | 20.5 (51)                                             |
| Cimetidine        | 14.2 ± 0.2 (98)                               | 38.5 ± 1.2 (77)                                         | 24.6 (69)                                             |

We also evaluated the ability of various additional organic cations to inhibit MPP⁺ uptake mediated by OCT3 (Table II). These organic cations were found to inhibit differentially the endogenous MPP⁺ uptake activity and the OCT3-specific MPP⁺ uptake activity. The potent inhibitors of OCT3-specific MPP⁺ uptake included dimethylamiloride, clonidine, guanidine, and TEA.

OCT3-mediated Uptake of Dopamine—The results showing the inhibition of OCT3-mediated MPP⁺ uptake by dopamine suggest but do not prove that this neurotransmitter is actually a transportable substrate for OCT3. To provide direct evidence for the transport of dopamine by OCT3, we compared the uptake of dopamine between vector-transfected and OCT3 cDNA-transfected HRPE cells (Fig. 4A). When the uptake was measured in mannitol medium, transfection of the cells with OCT3 cDNA led to a 85% increase in the uptake of dopamine. The OCT3-inducible uptake was demonstrable even when mannitol in the medium was isoosmotically replaced with N-methyl-D-glucamine chloride, sodium gluconate, or NaCl. Thus, the OCT3-specific uptake of dopamine was not dependent on Na⁺ and Cl⁻ (Fig. 4B). This clearly differentiates the OCT3-mediated uptake of dopamine from the uptake processes mediated by the (Na⁺, Cl⁻)-dependent dopamine and norepinephrine transporters (35, 34).

Characteristics of MPP⁺ Uptake in X. laevis Oocytes Expressing OCT3—We also studied the characteristics of OCT3-mediated uptake of MPP⁺ using the X. laevis oocyte expression system. Uptake of MPP⁺ was compared between un.injected oocytes and OCT3 cDNA-injected oocytes at different pH (data not shown). The uptake activity was severalfold higher in cDNA-injected oocytes than in control oocytes. The uptake was influenced by pH in both groups of oocytes. The endogenous uptake activity in control oocytes as well as the total uptake activity in OCT3-expressing oocytes increased significantly when the pH of the uptake medium was changed from 5.5 to 8.5. When OCT3-specific activity alone was considered, the pH-dependent changes were small in the pH range 5.5–7.5. However, a change of pH from 7.5 to 8.5 led to a 2-fold increase in the uptake activity.

Electrophysiological Characteristics of OCT3-mediated MPP⁺ Uptake—Our previous studies with OCT3 using TEA as the substrate showed that even though OCT3-mediated uptake was influenced by pH, the influence was entirely because of pH-induced changes in membrane potential in the oocyte (18). Electrophysiological studies subsequently confirmed that OCT3 is not an organic cation/proton antiporter but a potential-sensitive organic cation transporter (18). In the present study, we have corroborated these earlier data using MPP⁺ as the substrate. Superfusion of OCT3-expressing oocytes with MPP⁺ caused inward currents under voltage clamp conditions, indicating the electrogenic nature of the transport process, and the MPP⁺-induced currents were independent of extracellular pH. The influence of pH was not seen because the membrane potential was clamped. Using this two-electrode voltage-clamp technique, we have characterized the electrophysiologic aspects of MPP⁺ uptake mediated by OCT3 at pH 7.5. The currents induced by MPP⁺ were saturable with respect to MPP⁺, and membrane potential had significant influence on the sat-
Analysis of the saturation kinetics at three different testing membrane potentials showed that membrane potential influenced the apparent affinity of the transporter for its substrate as evidenced from the potential-dependent shift in the slope of the Eadie-Hofstee plots (Fig. 5B). The maximal velocity ($I_{\text{max}}$) of the transporter was also affected by membrane potential (Fig. 5C). The effect on $I_{\text{max}}$ was very small around the normal physiological membrane potential ($-30$ to $-70$ mV) but was much greater at hyperpolarizing membrane potentials. In contrast, the effect of membrane potential on $K_{0.5}$ for MPP⁺ (the concentration of MPP⁺ at which the induced current is 50% of the maximal current, $I_{\text{max}}$).

**Evidence for the Identity of OCT3 as the Extraneuronal Monoamine Transporter (uptake2)**—The studies described thus far have established the following. OCT3 is a potential-sensitive organic cation transporter, it transports the cationic neurotoxin MPP⁺ effectively, and it is capable of transporting the catecholamine dopamine in a Na⁺- and Cl⁻-independent manner. These characteristics are very similar to those described for the extraneuronal monoamine transporter, also called uptake2 (19–21). However, the molecular identity of this transporter has not yet been established.

The extraneuronal monoamine transporter has been shown to be responsible for the Na⁺- and Cl⁻-independent uptake of the monoamines (epinephrine, norepinephrine, dopamine, serotonin, and histamine) in several extraneuronal tissues. It is driven by the inside-negative membrane potential, and it interacts with a variety of organic cations, including MPP⁺. This raises the possibility that OCT3 may be identical to the extraneuronal monoamine transporter. Even though OCT1 and OCT2 possess some of the characteristics of the extraneuronal monoamine transporter, they are unlikely candidates because their expression is not widespread in mammalian tissues but is rather limited to kidney, intestine, and liver.

Therefore, we investigated the possibility that OCT3 may be identical to the extraneuronal monoamine transporter. A distinguishing characteristic of the extraneuronal monoamine transporter is its sensitivity to steroids (35, 36). To assess whether OCT3 possesses this characteristic, the ability of steroids to inhibit OCT3-mediated uptake of TEA, a prototypical organic cation, was studied in HRPE cells transfected with OCT3 cDNA (Fig. 6A). The endogenous TEA uptake activity in vector-transfected cells was $5\%$ of that in the cDNA-transfected cells. All steroids tested ($\beta$-estradiol, corticosterone, deoxycorticosterone, papaverine, testosterone, and progesterone) were found to inhibit TEA uptake markedly in cDNA-transfected cells. The catecholamines dopamine and norepinephrine also inhibited OCT3-mediated TEA uptake, but their inhibitory potency was much less compared with that of the steroids. The IC₅₀ values for the inhibition of OCT3 activity were then compared with the IC₅₀ values available in the literature (35–37) for the same steroids and catecholamines for the inhibition of the extraneuronal monoamine transporter activity (Table III). There was a significant correlation between the two sets of IC₅₀ (values $r = +0.88$) (Fig. 6B).

For comparison, similar experiments were carried out to assess the steroid sensitivity of the other two potential-sensitive organic cation transporters OCT1 and OCT2 in HRPE cells expressing the transporters heterologously. Transfection of the cells with OCT1 cDNA or OCT2 cDNA led to a 15- to 20-fold
increase in TEA uptake. The OCT1-mediated TEA uptake was not found to be sensitive to steroids (Fig. 7A). Even at a concentration of 100 μM, the steroids β-estradiol, corticosterone, deoxycorticosterone, and testosterone failed to inhibit OCT1 activity. Progesterone however inhibited OCT1 activity to a significant extent at this concentration. In contrast to OCT1, the transport activity of OCT2 was inhibited by all steroids tested (Fig. 7B). However, the order of inhibitory potency for OCT2 (progesterone, deoxycorticosterone, corticosterone, β-estradiol) was markedly different from that for OCT3 and extraneuronal monoamine transporter (β-estradiol, deoxycorticosterone, corticosterone, progesterone). In particular, the IC50 value for β-estradiol for the inhibition of OCT2 activity was 40 to 80 times greater than for the inhibition of OCT3 or extraneuronal monoamine transporter.

Table III
Relative affinities of steroids and catecholamines for interaction with OCT2, OCT3, and uptake2

| Inhibitor               | IC50 (μM) OCT3 | IC50 (μM) Uptake2 | IC50 (μM) OCT2 |
|-------------------------|---------------|------------------|--------------|
| β-Estradiol             | 1.1           | 1.8              | 84.8         |
| Corticosterone          | 4.9           | 2.6              | 4.2          |
| Deoxycorticosterone     | 8.4           | 5.3              | 1.9          |
| Progesterone            | 10.5          | 3.4              | 1.6          |
| Norepinephrine          | 434           | 245              | 11000        |
| Dopamine                | 384           | 500              | 2300         |

Because steroids have been shown to inhibit the activity of the extraneuronal monoamine transporter competitively (38), we studied the kinetic nature of the inhibition of OCT3 activity by steroids. The saturation kinetics of TEA uptake was examined in OCT3 cDNA-transfected HRPE cells in the absence and in the presence of β-estradiol (Fig. 8). The presence of β-estradiol had no significant effect on the kinetic parameters of TEA uptake. However, the presence of progesterone resulted in a decrease in the apparent Vmax and an increase in the apparent Km for TEA uptake. These results suggest that the inhibition of OCT3 activity by steroids is not mediated by a simple competitive inhibition mechanism.
In incubation) over the concentration range of 0.25–7.5 mM in the presence of corticosterone. The uptake of TEA in HRPE cells was determined by measuring TEA uptake (5-min incubation) over the concentration range of 0.25–7.5 mM in the presence of 2 µM β-estradiol. Results are given as Eadie-Hofstee plots. V, TEA uptake in pmol/10⁶ cells/5 min; S, TEA concentration in mM.

diol (2 µM) increased the Kᵣ value for TEA uptake 2.5-fold (1.20 ± 0.15 mM versus 3.18 ± 0.69 mM). There was no significant change in V_max (12.7 ± 0.8 versus 12.6 ± 1.8 pmol/10⁶ cells/5 min). These data show that β-estradiol is a competitive inhibitor of OCT3-mediated TEA uptake.

The inhibition of the extraneuronal monoamine transporter by steroids has been routinely studied using a catecholamine as the substrate for the transporter. Therefore, we assessed the ability of corticosterone to inhibit OCT3-mediated dopamine uptake (Fig. 9A). The uptake of dopamine in control cells was not inhibited by the steroid. In contrast, the uptake in OCT3 cDNA-transfected cells was inhibited. The inhibition of OCT3-mediated dopamine uptake was 60% at 10 µM corticosterone and 95% at 100 µM corticosterone (Fig. 9B). This inhibitory potency of OCT3-mediated catecholamine uptake was similar to that of OCT3-mediated TEA uptake as well as to that of catecholamine uptake mediated by the extraneuronal monoamine transporter.

Evidence for Expression of OCT3 in the Brain—Because our studies have clearly shown that OCT3 interacts with various cationic neurotoxins and neurotransmitters, a possibility arises that this transporter may play a significant role in the handling of these cationic compounds in the brain. Therefore, we sought to obtain evidence for the expression of OCT3 in the brain. Northern blot analysis of poly(A)¹⁺ RNA isolated from various rat tissues showed that OCT3-specific transcripts are present in a number of tissues including brain (Fig. 10). This indicates that OCT3 is expressed in the central nervous system. It is to be noted however that OCT3 is not the only cloned organic cation transporter that interacts with cationic neurotoxins and neurotransmitters. OCT1 and OCT2 are also known to transport MPP⁺ and other neuroactive organic cations (10, 11, 15–17). Therefore, we assessed the possible expression of these organic cation transporters by sequentially hybridizing the same membrane blot used in the analysis of OCT3 expression with OCT1 and OCT2 cDNA probes (Fig. 10). OCT1-specific transcripts were detectable primarily in kidney and liver and to a much smaller extent in intestine. In contrast, OCT2-specific transcripts were detectable only in kidney. But, neither OCT1-specific transcripts nor OCT2-specific transcripts were seen in brain. The same membrane blot was also probed with glyceraldehyde 3-phosphate dehydrogenase cDNA to provide evidence for the presence of RNA in each lane (Fig. 10). Thus, among the three potential-sensitive organic cation transporters cloned to date, only OCT3 is expressed in brain.

To confirm the identity of the mRNA species hybridizing to the OCT3 cDNA probe, we performed RT-PCR using OCT3-specific primers and rat brain poly(A)⁺ mRNA. Rat placental poly(A)⁺ mRNA was used as a positive control. Both RNA samples yielded a similar size RT-PCR product (0.85 kbp) expected from the positions of the primers in OCT3 cDNA (data not shown). These two RT-PCR products were gene-cleaned and subjected to restriction site analysis using three different enzymes (PvuII, SaeI, and TaqI). The restriction pattern with all three enzymes was identical for the RT-PCR products from placenta and brain and was exactly as expected from the
known restriction map of rat OCT3 cDNA (data not shown). These data confirm that the RT-PCR product from rat brain is indeed identical to OCT3.

To provide further evidence for the expression of OCT3 in brain, we screened a rat brain cDNA library with a 1.4-kbp fragment of rat OCT3 cDNA as the probe. This screening resulted in a single positive clone. Sequencing of this clone showed that it was identical to rat OCT3 (data not shown). Taken collectively, these data provide unequivocal evidence for the expression of OCT3 in rat brain.

Regional Distribution of OCT3-specific Transcripts in Brain—In situ hybridization with OCT3-specific antisense riboprobe to sagittal brain sections revealed widely distributed expression (Fig. 11, A (hematoxylin-eosin staining) and B (antisense probe)). Significant levels of a speckled distribution pattern of OCT3 transcripts were observed throughout the cerebral cortex and the pontine nucleus. Intense labeling was seen in the cerebellum (Fig. 11, C and D), and moderate labeling was seen in the hippocampus (Fig. 11E). In the cerebellum, prominent expression was detected in the single layer of huge Purkinje cells and in the inner granular cell layer. Significant levels of expression were also detectable in the deep cerebellar nuclei. There was no significant expression in the molecular layer nor in the white matter. In the hippocampus, expression was evident in the cornu ammonis pyramidal neurons, granular cells of the dentate gyrus as well as...
interneurons. The sense probe did not yield positive signals under identical experimental conditions anywhere in the brain (Fig. 11F), demonstrating the specificity of the signals seen with the antisense probe.

**DISCUSSION**

This is the first report on the expression and regional distribution of an organic cation transporter in the brain. Of the three potential-sensitive organic cation transporters cloned and characterized thus far, only OCT3 is expressed in the brain. Evidence for the expression of OCT3 in the brain has been obtained in the present study by different experimental approaches, which include Northern blot analysis, RT-PCR, and *in situ* hybridization. Unequivocal evidence for the identity of the mRNA transcript detected by these approaches comes from successful cloning of the OCT3 cDNA from a brain cDNA library. The present study also demonstrates for the first time that OCT3 is capable of transporting various cationic neurotoxins and neurotransmitters. The neurotoxin MPP⁺ is an excellent substrate for OCT3, with a *K* value in the μM range. The ability of OCT3 to transport MPP⁺ in heterologous expression systems is demonstrable by direct uptake of radiolabeled MPP⁺ as well as by membrane depolarization induced in the presence of unlabeled MPP⁺. The *K* value for OCT3-mediated uptake of MPP⁺ is 91 ± 10 μM in HRPE cells heterologously expressing OCT3. However, when studied in OCT3-expressing *X. laevis* oocytes, the *K* value is considerably lower. With the membrane potential clamped at −50 mV, the *K* value in oocytes is 24 ± 2 μM. This value decreases significantly if the membrane potential is hyperpolarized relative to −50 mV but increases if the membrane potential is depolarized relative to −50 mV. Uptake measurements in HRPE cells were done without clamping the membrane potential, and this might be the most likely reason for the difference in the *K* values between the two expression systems. Competition experiments show that the abusable drugs amphetamine and methamphetamine and the neurotransmitters dopamine and serotonin also interact with OCT3 with appreciable affinity. In addition, the actual transport of dopamine via OCT3 is demonstrable in OCT3-expressing cells by measuring the uptake of radiolabeled dopamine. The ability of OCT3 to interact with cationic neurotoxins and neurotransmitters and the expression of OCT3 in the brain suggest that OCT3 is likely to play a significant role in the handling of these neuroactive compounds in the brain. *In situ* hybridization studies show that OCT3 is widely expressed in different regions of the brain including cerebral cortex, cerebellum, and hippocampus. The monoamine neurotransmitters (dopamine, serotonin, and norepinephrine) do not appear to be the common denominator for these OCT3-expressing brain regions. Therefore, the physiological function of OCT3 in the brain remains yet to be identified. On the other hand, OCT3 is likely to play a role in the disposition of MPP⁺, amphetamines, and other cationic neurotoxins in OCT3-positive regions of the brain. OCT3 is driven by membrane potential, and therefore this transporter potentially mediates the membrane potential-dependent concentrative accumulation of these neurotoxins into the cells. This may have relevance to the pharmacological actions of these neuroactive agents.

OCT1 and OCT2 are known to transport MPP⁺ and monoamines (10, 11, 15–17). Sensitive RT-PCR studies have indicated that OCT1- and OCT2-specific transcripts are expressed in the brain (10, 14), but the transcripts are not detectable by Northern blot analysis (8, 10, 11, 13). In comparison, OCT3-specific transcripts are easily detectable in the brain by Northern blot analysis. However, a recent study has provided convincing evidence for the expression of OCT2 in the brain using *in situ* hybridization and immunocytochemistry (39).

An important finding in the present study, which is of significant pharmacological and physiological relevance, is the identification of OCT3 as the extraneuronal monoamine transporter. Even though the extraneuronal monoamine transporter is known to transport its physiological substrates such as norepinephrine and dopamine with low affinity, the relevance of this transporter to the disposition and metabolism of these monoamines has been well established (40–42). The establishment of the molecular identity of this transporter as OCT3 strongly suggests that this transporter is likely to play a significant role in the disposition and metabolism of monoamines in the brain. Most of the functional studies involving this transporter have been done in peripheral tissues. However, recent studies have provided evidence for the expression of the extraneuronal monoamine transporter in the central nervous system (43–46). The present study demonstrating the widespread expression of OCT3 in the brain corroborates these findings. In addition to the physiological function of OCT3 in brain and in peripheral tissues in the uptake and metabolism of monoamines, this transporter may also have therapeutic potential in the treatment of malignant brain tumors. Noe et al. (45) have recently shown that the extraneuronal monoamine transporter expressed in a human glioma cell line mediates the accumulation of the cytotoxic drug (2-chloroethyl)-3-sarcosinamide-1-nitrosourea. This drug is one of the most active agents for the treatment of malignant astrocytomas (47, 48). The importance of the extraneuronal monoamine transporter to the therapeutic action of this drug is evident from the observations that the sensitivity of the target cell lines to the drug correlates with the expression levels of the transporter (45, 49, 50). Therefore, OCT3 in the brain has the therapeutic potential in facilitating the accumulation of this and other structurally related cytotoxic drugs in malignant tumors of the central nervous system.

Recently, two additional organic cation transporters have been cloned (51, 52). These transporters, OCTN1 and OCTN2, are expressed widely in human tissues, including the brain. Based on amino acid sequence homology, OCTN1 and OCTN2 form a distinct subgroup within the organic cation transporter family. Unlike OCT1, OCT2, and OCT3, which have been unequivocally shown to be potential-sensitive transporters, the role of membrane potential in the transport function of OCTN1 and OCTN2 has not been investigated. Furthermore, very little is known at present regarding the substrate specificity of these two transporters. OCTN1 transports tetraethylammonium, but no additional information is available on its interaction with other organic cations (51). OCTN2 also transports tetraethylammonium, and it does interact with other organic cations including the neurotoxin MPP⁺ (52). However, the substrate specificity of OCTN2 is clearly distinct from that of OCT3. For instance, amphetamine does not appear to interact with OCTN2, whereas OCT3 interacts with amphetamine with high affinity. Recently it has been shown that OCTN2 can mediate the transport of carnitine in a Na⁺-dependent manner (53). It is likely that OCTN1 and OCTN2 also interact with cationic neurotoxins and neurotransmitters, although with differing affinity and specificity. The regional distribution of these two transporters in the brain remains yet to be established.

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