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Role of Organic Cation Transporters in Dopamine Uptake across Olfactory and Nasal Respiratory Tissues

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Abstract: Dopamine has poor oral bioavailability and low permeability across the blood–brain barrier, and yet it has been reported to have good systemic and central nervous system (CNS) bioavailability following nasal administration. The aim of this study was to investigate the extent to which dopamine transport in the olfactory and respiratory mucosae results from the activity of organic cation transporters (OCTs) present in the nasal cavity. Transport studies were carried out to determine the mechanism of dopamine transport across bovine olfactory and nasal respiratory mucosa. Western blotting and immunohistochemistry were performed to determine the expression and localization of organic cation transporter-2, a major transporter of dopamine, in the nasal mucosa. Dopamine transport was found to be saturable across both tissues. Amantadine, an organic cation transporter-1 (OCT-1) and organic cation transporter-2 (OCT-2) mixed inhibitor, decreased dopamine flux to a greater extent than guanidine, a more specific organic cation transporter-2 inhibitor. Immunohistochemistry results showed that organic cation transporter-2 was localized in both the epithelial and submucosal regions of the nasal olfactory and respiratory mucosa. Dopamine transport across the olfactory and respiratory mucosae is partially mediated by organic cation transporters, including OCT-1 and OCT-2. Utilization of uptake transporters may provide the opportunity for improved systemic absorption and targeted CNS delivery of dopamine and other drug compounds following nasal administration.

Keywords: Nasal absorption; dopamine; organic cation transporter; olfactory transport; respiratory transport

Introduction

Dopamine is a catecholamine neurotransmitter subject to extensive hepatic metabolism following oral administration. It is essentially completely ionized at physiological pH, which results in its poor permeation across the blood–brain barrier (BBB) and other cell membranes. Enhanced uptake of dopamine into the central nervous system (CNS) of rats and mice following nasal administration has been reported by Dahlin and co-workers.[1,2] These investigators observed significantly higher amounts of dopamine in the CSF, cerebellum, cerebrum, and left and right olfactory bulbs of rats and mice following nasal compared to iv administration. Similar observations of enhanced dopamine CNS bioavailability following intranasal administration have also been reported in other species, including rhesus monkeys and beagle dogs.[3,4] Because of the low permeability of dopamine across epithelial and endothelial cells, it was hypothesized that active transporters must be responsible for the uptake

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of dopamine from the nasal mucosa, enabling its subsequent distribution into the CNS.

Within the CNS, dopamine is preferentially transported intraneuronally by the dopamine transporter (DAT)[5] while peripherally, dopamine is transported primarily by the family of organic cation transporters (OCTs).[6] While OCT-1, OCT-2, and OCT-3 are known to transport dopamine, OCT-2 is reported to have a higher affinity for dopamine than OCT-1,[7] while the kinetics of OCT-3 transport of dopamine have not been reported. Thus, the characterization of the role and localization of OCT-2 in the nasal mucosa was the focus of these investigations.

The OCTs are facilitative transporters belonging to the solute carrier superfamily of transporters. They are membrane-spanning proteins composed of 12 transmembrane domains with both the amino and carboxy terminals on the cytoplasmic side. OCT-1, OCT-2, and OCT-3 are known to be responsible for the clearance of organic cations in the kidneys, liver, and intestines, and they have been shown to facilitate the transport of many of the catecholamine neurotransmitters[7,8] and drug substrates such as quinidine, probenecid, memantine, and guanidine.[6,8–12] OCT-1 has been observed on the basolateral membrane of the intestinal and renal epithelia.[6,8,13] OCT-3 has been observed in the kidneys, placenta, and other extraneuronal tissues.[14,15] OCT-2, while also expressed on the basolateral surface of the renal tubules, has lower expression in the intestinal epithelium.[13] With regard to the nasal mucosa, Monte et al., while investigating OAT activity in the murine olfactory mucosa, showed that OCT-1 and OCT-2 were present in this tissue.[16] Typical K_m values for dopamine transport by OCT-2 are in the millimolar range.[6,9,10]

After previous demonstration of the expression, localization, and role of DAT in dopamine transport across the nasal tissues,[17] the objective of these studies was to investigate the role of OCTs in dopamine transport at higher substrate concentrations, which would be more relevant to the exogenous nasal doses used by Dahlin et al.[1,2] Bovine olfactory and respiratory mucosae were used in these studies because their structures and organization bear close resemblance to the structure of the human nasal mucosa,[18–20] and discrete samples of both tissues can be readily obtained from donor cattle.[21–23]

Experimental Section

All chemicals, including dopamine HCl, amantadine HCL, and guanidine HCl, were obtained from Sigma-Aldrich

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Chemical Co. (St. Louis, MO). Krebs Ringers buffer (KRB) contained 0.49 mM $MgCl_2$, 4.56 mM KCl, 120 mM NaCl, 0.70 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 10 mM dextrose, 2.52 mM $CaCl_2$, and 84 mM $NaHCO_3$. KRB–sodium metabisulfite solution contained KRB with 0.05 mg/mL $Na_2S_2O_5$, which was added to limit the atmospheric oxidation of dopamine. Nasal mucosal tissues were obtained from local abattoirs (Roehrkassee Meat Lockers, Williamsburg, IA, and Bud's Custom Meats Co, Riverside, IA) and transported to the laboratory in ice-cold Krebs–Ringer buffer (KRB).

Transport Studies. Mucosal explants (2 cm^2) were mounted between the donor and receiver chambers of Snapwell diffusion chambers (Harvard Apparatus, Holliston, MA), and excess tissue was trimmed away. The explants were equilibrated for 20–30 min with 6 mL of buffer (KRB + sodium metabisulfite (0.05 mg/mL)) or buffer + inhibitor (for inhibitor studies) in both the donor and receiver chambers. After equilibration, the buffer was replaced by donor (buffer + drug or buffer + drug + inhibitor) and receiver (buffer or buffer + inhibitor) chamber solutions. The tissues were kept aerated with carbogen (95% O_2 + 5% CO_2), and the temperature was maintained at 37 °C throughout the experiment.

Transport studies were conducted in the mucosal–submucosal direction, and samples (400 μL) were withdrawn from the receiver chamber at regular intervals and replaced by fresh, prewarmed buffer solution for a period of up to 120 min. The withdrawn samples were analyzed for dopamine content by HPLC. The cumulative amount of dopamine transported across each tissue was calculated and used to quantify the flux of dopamine at each fixed donor concentration.

Tissue Integrity. Transport studies were completed within approximately 4 h of harvest of the nasal tissues, and it has previously been established that these tissues typically remain viable for at least this period of time.[20–23] Lucifer yellow (LY), a paracellular transport marker, was used to verify the integrity of the explants by adding it in a sufficient quantity to the donor chamber at the end of each transport study to result in a concentration of ~200 μg/mL. The LY concentration in the receiver chamber was measured after 1 h using fluorescence spectroscopy (Kontron SFM25, Kontron Instruments, Everett, MA) with a $\lambda_{excitation}$ of 420 nm and $\lambda_{emission}$ of 530 nm. Transport of more than 0.1% of LY into the receiver chamber was considered to be a sign of damage to the tissues, and transport data from these tissues were discarded. Transepithelial electrical resistance (TEER) measurements were also performed before and after each study as well as at two time points during the study (EVOM, World Precision Instruments, Inc., Sarasota, FL). A decrease of >20% in the initial TEER value was considered significant, and results from such studies were discarded.

Dopamine flux was calculated using Fick's first law by dividing the slope of the steady-state portion of the cumulative amount of drug transported vs time curve by the tissue area exposed to the donor phase (1.13 cm^2). The permeability was obtained by dividing the flux by the initial concentration of dopamine in the donor chamber (2.1, 3.2, 4.2, 5.3, 10.2, and 21.1 mM).

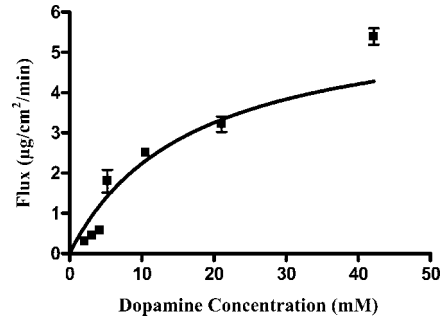

Figure 1. Mucosal–submucosal flux of dopamine across the bovine olfactory mucosa. Transport studies were carried out for 120 min, and flux was calculated using Fick's first law. Flux was saturable and yielded a K_m of 16.8 mM and J_{max} of 6.0 μg cm^{-2} min^{-1} when a Michaelis–Menten equation was fit to the mean data (GraphPad Prism 4.0 software, GraphPad Software Inc., San Diego, CA). Mean flux ± standard deviation values are shown; n = 3–4 (some deviations are within the symbol).

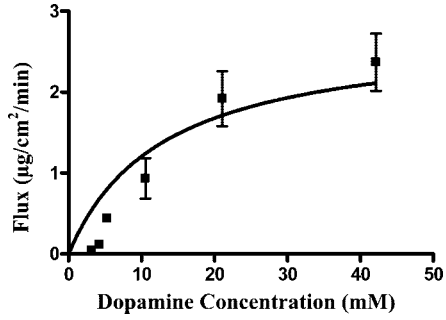

Figure 2. Mucosal–submucosal transport of dopamine across the bovine respiratory mucosa. Transport studies were carried out for 120 min, and flux was calculated using Fick's first law. Flux was saturable and yielded a K_m of 18.4 mM and J_{max} of 2.7 μg cm^{-2} min^{-1} when a Michaelis–Menten equation was fit to the mean data (GraphPad Prism 4.0 software, GraphPad Software Inc., San Diego, CA). Mean flux ± standard deviation values are shown; n = 5–10 (some deviations are within the symbol).

Transport Inhibition. The effect of amantadine (0.1 mM)[8] and guanidine (1 mM),[10] both OCT-2 inhibitors, on dopamine transport was studied by equilibrating the explants with buffer + inhibitor for 30 min prior to the initiation of the transport study. Transport studies were carried out as described above except the inhibitors were included in the donor and receiver solutions used during the study.

HPLC Analysis. The HPLC system used to measure dopamine concentrations consisted of a Shimadzu LC-600 pump, a Waters 712 autoinjector (Waters Inc., Milford, MA) and a Shimadzu SPD-6A UV–vis detector (280 nm) interfaced with a Shimadzu C-R4A Chromatopac integrator system (Shimadzu Scientific Instruments, Columbia, MD).

The mobile phase contained acetonitrile (17%) and water containing 1% acetic acid, 0.5 mM disodium EDTA, and 2.5 mM sodium octyl sulfonate. A Phenomenex Luna 5 μm, C-18, 250 mm $\times$ 4.6 mm column (Phenomenex Inc., Torrance, CA) was used with a mobile phase flow rate of 1 mL/min.

Western Blotting. Tissue segments were rinsed with ice-cold KRB and homogenized with KRB containing protease inhibitors (Sigma protease inhibitor kit, catalog no. P8340, Sigma Diagnostics Inc., St. Louis, MO). Rat kidney extract, known to express OCT-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), was used for a comparison to a known expressed protein. The homogenate was centrifuged at 1000*g* for 15 min at 4 °C; the supernatant was decanted and centrifuged at 10000*g* for 30 min at 4 °C. The supernatant was decanted once again and centrifuged at 100000*g* for an additional 1 h at 4 °C. The pellet was solubilized by heating in loading buffer (4.5 g of sodium dodecyl sulfate, 1.69 g of Tris base, 15 g of sucrose, 0.46 g of dithiothreitol, 6 mg of bromophenol blue, and distilled water to 50 mL) at 100 °C for 5 min. The samples were separated on 10% SDS–polyacrylamide (PAA) gels (Biorad, Hercules, CA), and the proteins were transferred to nitrocellulose membranes (Biorad, Hercules, CA). Nonspecific binding was blocked with blocking buffer (5% nonfat milk in 0.15% v/v Tween-20, 10 mM Tris base, and 100 mM NaCl) for 60 min. The membranes were incubated with primary antibody (rabbit anti-rat OCT-2, 1:500 diluted with blocking buffer; Alpha Diagnostics International Inc., San Antonio, TX) for 1 h. The membranes were rinsed with wash buffer (0.15% v/v Tween-20, 10 mM Tris base, and 100 mM NaCl for 15 min) followed by incubation with a horseradish peroxidase conjugated goat anti-rabbit secondary antibody (1:20000 diluted with blocking buffer, donated by Dr. Craig Morita, Department of Immunology, The University of Iowa). The immunoreactive bands were rinsed with wash buffer and developed using an enhanced chemiluminescence (ECL) kit (Pierce Biotechnology, Rockford, IL).

Immunohistochemistry. Bovine nasal explants were cryofrozen in liquid nitrogen, and 10 μm thick sections were cut, placed on slides, and dried overnight at 4 °C. The sections were brought to room temperature and rinsed with phosphate buffered saline (PBS) for 2 min. The sections were washed with distilled water for 2 min and treated with quenching solution (0.3% H_2O_2) followed by two 1 min rinses with distilled water. Sections were incubated with the primary antibody (rabbit anti-rat OCT-2 antibody, 1:5000, Alpha Diagnostics International, CA) overnight. The sections were rinsed with PBS twice (5 min each) and then treated with HRP conjugated secondary antibody (1:10000 dilution, goat anti-rabbit) for 30 min followed by two 5 min rinses with PBS. This was followed by treatment with a solution of hydrogen peroxide and alkaline phosphatase (ScyTel Laboratories, Logan, UT) using Nuclear Fast Red (ScyTel Laboratories, Logan, UT) as the reactive chromogen, which imparts a red coloration to the antigen–antibody complex. To improve the visualization of the red color, the sections were also counterstained with hematoxylin. The dye was rinsed away with 100% ethanol for 1 min followed by two butanol rinses each for 1 min. This was followed by two 2 min rinses with xylene. The final sections were coverslipped and examined using an Olympus BX 51 microscope. Additional sections were subjected to the same treatment as described above; however, instead of exposure to the primary antibody against OCT-2, they were treated with a nonspecific rabbit IgG to identify nonspecific binding that may have been due to the IgG moeity.

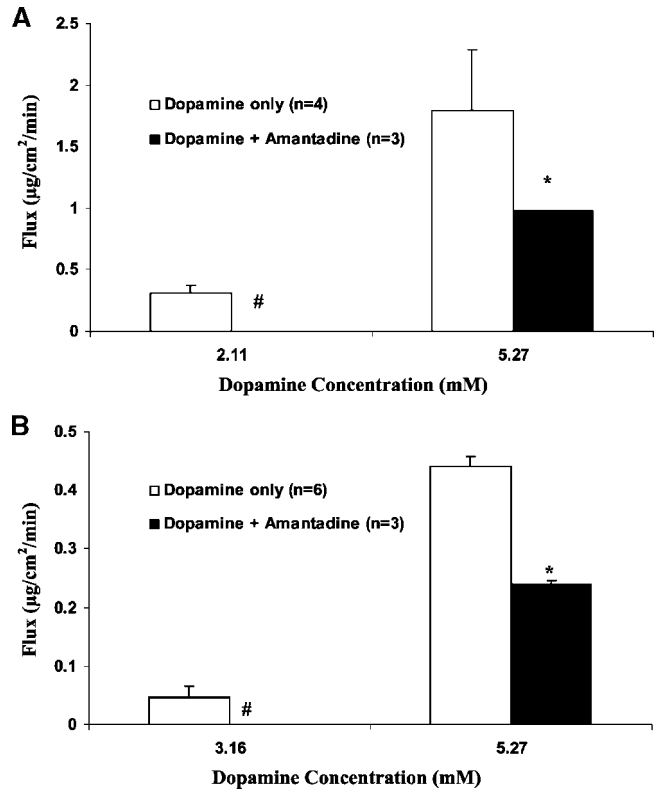

Figure 3. Effect of an OCT-1/OCT-2 mixed inhibitor, amantadine (100 μM), on mucosal–submucosal dopamine flux across bovine explants. Dopamine transport across (A) olfactory mucosa and (B) respiratory mucosa was measured over a 120 min period. Separate experiments were conducted using the same dopamine concentration and included 100 μM amantadine in both the donor and receiver chambers. Mean flux $\pm$ standard deviation values are shown (some deviations are within the symbol). Unpaired two tailed *t* tests ($p < 0.05$) were performed to compare the mean flux values at each dopamine concentration in the presence and absence of inhibitor. A significant difference in flux compared to the control ($p < 0.05$) is indicated by an asterisk ($*$). The symbol # indicates that dopamine flux was not measurable.

Results

Mucosal Transport. The flux of dopamine in the mucosal–submucosal (m–s) direction across both the

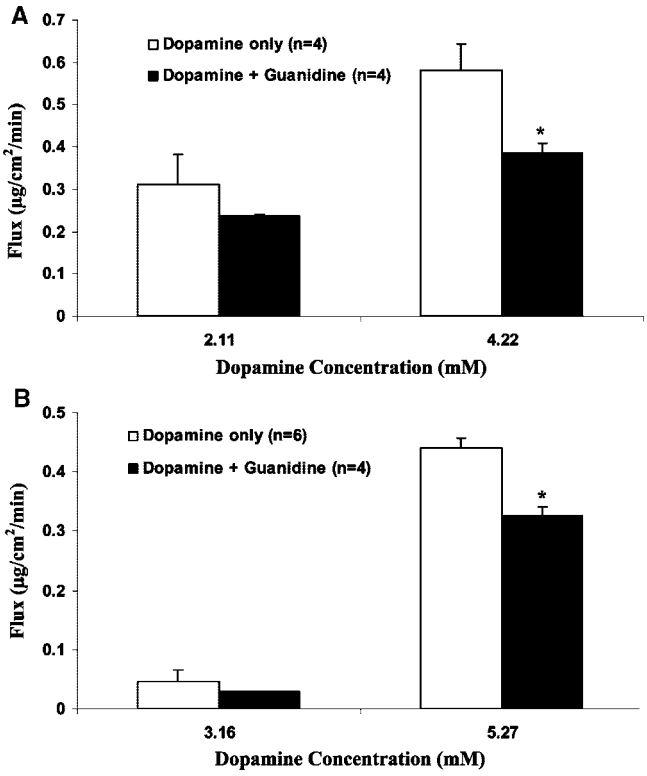

Figure 4. Effect of an OCT-2 inhibitor, guanidine (1 mM), on mucosal–submucosal dopamine flux across bovine olfactory explants. Dopamine transport across(A) olfactory mucosa and (B) respiratory mucosa was measured over a 120 min period. Separate experiments were conducted using the same dopamine concentration including 1 mM guanidine in both the donor and receiver chambers. Mean flux ± standard deviation values are shown. Unpaired two tailed *t* tests ($p < 0.05$) were performed to compare the mean flux values at each dopamine concentration in the presence and absence of inhibitor. A significant difference in flux compared to the control ($p < 0.05$) is indicated by an asterisk (∗).

olfactory (Figure 1) and nasal respiratory (Figure 2) mucosae increased in a nonlinear manner with increasing dopamine donor concentration. Fitting a Michaelis–Menten (M–M) type equation (GraphPad Prism 4.0, GraphPad Software Inc., San Diego, CA) to the flux results gave a J_{max} of 6.0 μg cm^{-2} min^{-1} and a K_m of 16.8 mM across the olfactory mucosa (Figure 1). Fitting a Michaelis–Menten (M–M) type equation to the nasal respiratory m–s results gave a J_{max} of 2.7 μg cm^{-2} min^{-1} and a K_m of 18.4 mM (Figure 2). While the data show some deviation, especially at low and high concentrations, from the single-carrier model assumed by the M–M fitting, the saturable transport observed strongly suggests that at least one transporter and probably several transporters are active in the concentration range tested. The calculated K_m and V_{max} parameters are likely influenced by all of the carrier activities and, as a result, should not be used as the only confirmatory test for the presence of OCT-2.

To further investigate the role of OCTs in dopamine flux, two OCT inhibitors, amantadine and guanidine, were included in additional transport studies. For these studies, the dopamine concentrations investigated were below the observed K_m values, ensuring that inhibition could be clearly observed. Unpaired, two-tailed *t* tests were performed to compare the mean of the dopamine flux values obtained at each dopamine donor concentration in the presence and absence of inhibitor. Amantadine (100 μM), which inhibits both OCT-1 and OCT-2, produced a significant decrease in dopamine flux in the m–s direction across both the olfactory mucosa and nasal respiratory mucosae (parts A and B of Figure 3, respectively). Guanidine (1 mM), an OCT-2, OCT-3, and PMAT (plasma membrane monoamine transporter) inhibitor, decreased dopamine olfactory flux in the m–s direction at 2.11 and 4.22 mM dopamine (Figure 4A), while across the respiratory mucosa, guanidine produced a significant decrease in dopamine flux in the m–s direction at all concentrations investigated (Figure 4B). At low dopamine concentrations (2.11 mM), amantadine reduced the dopamine flux to below the level of detection compared to the 23% decrease in dopamine flux across the olfactory mucosa produced by guanidine. Across the respiratory mucosa, dopamine (3.16 mM) flux was not detected in the presence of amantadine, while the flux was reduced by 40% in the presence of guanidine.

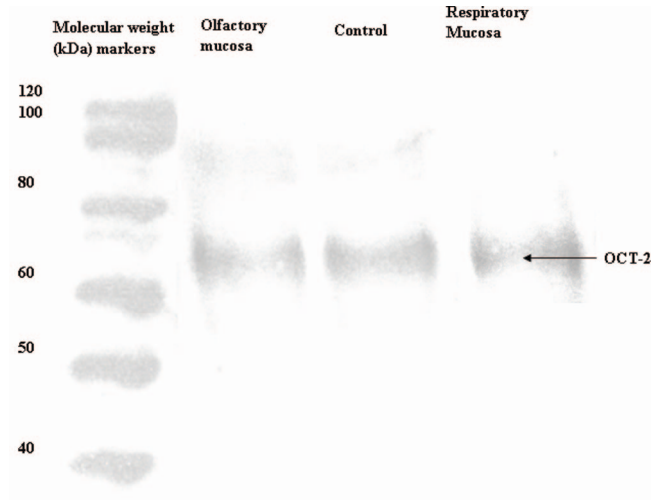

Figure 5. Determination of OCT-2 expression in bovine olfactory and respiratory mucosae by Western blotting. Membrane fractions from olfactory and respiratory explants were separated on PAA gels and treated with rabbit anti-rat antibody against OCT-2. HRP conjugated goat anti-rabbit secondary antibody was used, and the reactive band was visualized by chemiluminescence. A band between 60 and 80 kDa was observed that was similar to that of the control band (rat kidney extract) known to express OCT-2.

Western Blotting. Western blots were produced using an anti-rat, OCT-2 specific antibody, and the expression of bOCT-2 was observed as bands with molecular weight between 60 and 70 kDa (Figure 5). The lane containing the rat kidney extract preparation also showed a similar

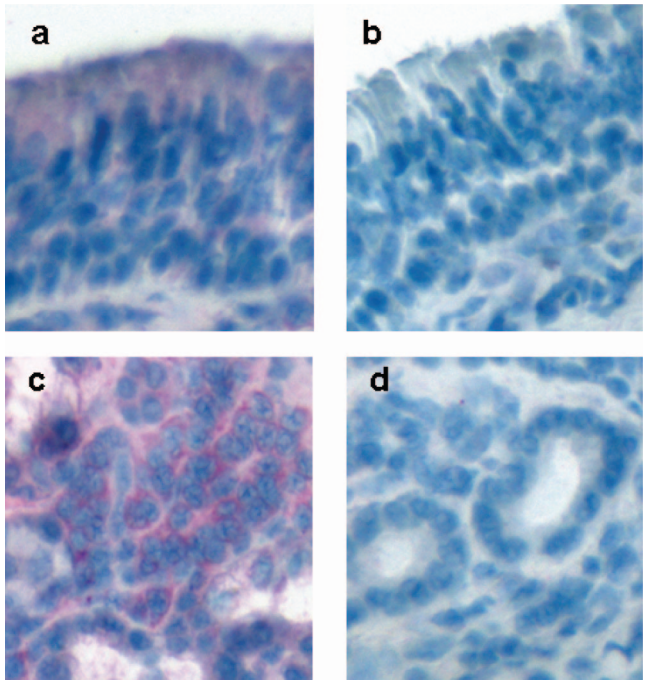

Figure 6. OCT-2 localization by immunohistochemistry in bovine olfactory mucosa. Sections of bovine olfactory mucosa were treated with rabbit anti-rat anti-OCT-2 antibody and goat anti-rabbit secondary antibody. Control olfactory mucosal sections were treated with an irrelevant rabbit anti-rat IgG and goat anti-rabbit secondary antibody to detect nonspecific binding. The sections were counterstained with hematoxylin and viewed using an Olympus BX 51 microscope: (a) the epithelial cell layer of the olfactory mucosa, where the presence of OCT-2 is observed by the red coloration in the apical region of the epithelial cells; (b) control olfactory tissue highlighting the epithelial region; (c) submucosal glandular region of the olfactory mucosa, where prominent OCT2 labeling is observed surrounding the glandular epithelium; (d) control olfactory submucosal tissue highlighting a region containing submucosal glands.

band at 60–70 kDa. These results correlate well with the reported molecular weight of OCT-2 observed in renal tissues.[24]

Immunohistochemistry. Light microscopy showed that the olfactory and respiratory mucosae contained a ciliated columnar epithelial layer overlying an extensive submucosal region (Figures 6 and 7). The submucosal regions contain nasal glands and blood vessels within a connective tissue matrix. When compared to the tissues exposed to the irrelevant IgG (Figure 6b,d), the red coloration in the epithelial and submucosal regions of the olfactory mucosa (Figure 6a,c) suggests the presence of bOCT-2. Compared

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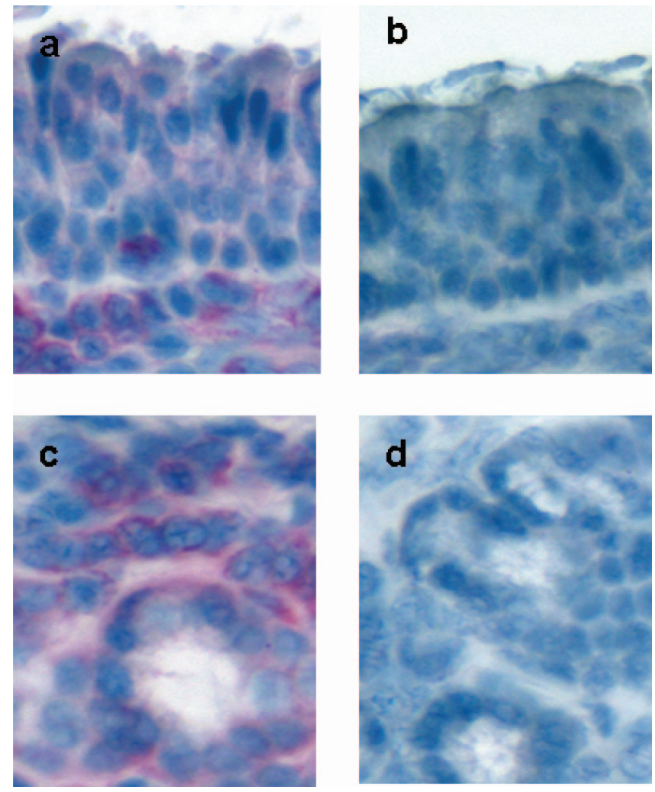

Figure 7. OCT-2 localization by immunohistochemistry in bovine respiratory mucosa. Sections of bovine olfactory mucosa were treated with rabbit anti-rat anti-OCT-2 antibody and goat anti-rabbit secondary antibody. Control respiratory mucosal sections were treated with an irrelevant rabbit anti-rat IgG and goat anti-rabbit secondary antibody to detect nonspecific binding. The sections were counterstained with hematoxylin and viewed using an Olympus BX 51 microscope: (a) the epithelial cell layer of the respiratory mucosa, where the presence of OCT-2 is observed by the red coloration in the apical region of the epithelial cells and on the plasma membrane surrounding the basal cells; (b) control respiratory tissue highlighting the epithelial region; (c) submucosal glandular region of the respiratory mucosa, where prominent OCT2 labeling is observed surrounding the glandular epithelium; (d) control olfactory submucosal tissue highlighting a region containing submucosal glands.

to the irrevelant IgG controls (Figure 7b,d), the red coloration in the epithelial and submucosal regions of the respiratory mucosa (Figure 7a,c) also suggests the presence of bOCT-2 in the respiratory mucosa. There was little difference observed in the pattern of bOCT-2 distribution between the olfactory and respiratory mucosae.

Discussion

Three OCTs (OCT-1, OCT-2, and OCT-3) are known to transport dopamine, and all share similar substrates although with different specificities. The expression of both OCT-1 and OCT-2 in the murine olfactory mucosa by RT-PCR was

recently reported,[16] providing evidence that both of these transporters are present in the nasal mucosa. It has also been reported that dopamine has some affinity for the NET (norepinephrine) and SERT (serotonin) transporters.[6] The deviations of the measured flux from that predicted by the M–M fitting in the respiratory mucosa (Figure 2) and at high concentrations in the olfactory mucosa (Figure 1) suggest that processes in addition to OCT-mediated transport are playing a role in dopamine uptake in these tissues. The activities of the OCTs and the neurotransmitter transporters, including DAT,[17] NET, and SERT, may influence the total dopamine transport. Since several of these are facilitative transporters present at various cellular and submucosal locations in these tissues, they may be acting as influx or efflux transporters for dopamine at the concentrations used in these studies, thus explaining both the positive and negative deviations when using a simplified, single-carrier uptake model.

When OCT inhibitors were included in the transport studies, amantadine, an OCT-1 and OCT-2 inhibitor, produced a more marked decrease in dopamine flux compared to guanidine, an agent capable of inhibiting OCT-2, OCT-3, and PMAT (Figures 3 and 4). Yet complete inhibition of transport was still not observed, especially at high donor concentrations. Since passive transport of dopamine is likely to be negligible, even at higher donor concentrations, the observed transport of dopamine is likely the result of multiple additional transporters, including DAT, OCT-1, and OCT-2 transferring dopamine across the tissue explants.

OCT-2 localization in epithelial tissues including a substantial submucosal layer has not been previously reported. The immunohistochemistry results suggest that bOCT-2 is associated with the nasal submucosal structures of the bovine explants, with some bOCT-2 also observed in the epithelial region. The significant submucosal presence of OCT-2 suggests that it may assist in the transport of dopamine into or from the nasal glands and blood vessels. The submucosal localization of OCT-2 also suggests a detoxifying role for OCT-2 in the nasal mucosa, similar to that ascribed to OCT-2 in the kidneys. Regardless of its endogenous role, the demonstration of the presence and activity of OCT-2 in the full-thickness nasal mucosal explants suggests that ascribing all transporter behavior to the epithelial cells, as is commonly done in the interpretation of cell culture results, may lead to erroneous conclusions about the role of a particular transporter at a distinct anatomical location or within a specific organ system.

The presence of numerous transporters for dopamine (as well as for other neurotransmitters) highlights the pivotal role played by these transporters in maintaining well-controlled concentrations of dopamine at various sites within the body. These transporters represent important potential pathways for the uptake of exogenous compounds across the nasal mucosa and, potentially, into the brain. The observation that dopamine is preferentially distributed into the brain following nasal administration provides strong evidence that transporters within the nasal mucosa can be exploited to target drug delivery from the nasal cavity into the brain.

Abbreviations Used

OCT, organic cation transporter; OCT-1, organic cation transporter-1; bOCT-1, bovine organic cation transporter-1; OCT-2, organic cation transporter-2; bOCT-2, bovine organic cation transporter-2; DAT, dopamine transporter; CNS, central nervous system; BBB, blood–brain barrier; CSF, cerebrospinal fluid.

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