Full Paper

Expression of Human Organic Anion Transporters in the Choroid Plexus and Their Interactions With Neurotransmitter Metabolites

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Abstract. The purpose of the present study was to elucidate the expression of human organic anion transporter 1 (hOAT1) and hOAT3 in the choroid plexus of the human brain and their interactions with neurotransmitter metabolites using stable cell lines. Immunohistochemical analysis revealed that hOAT1 and hOAT3 are expressed in the cytoplasmic membrane and cytoplasm of human choroid plexus. Neurotransmitter metabolites, namely, 5-methoxyindole-3-acetic acid (5-MI-3-AA), homovanillic acid (HVA), vanilmandelic acid (VMA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindole-3-acetic acid (5-HI-3-AA), *N*-acetyl-5-hydroxytryptamine (NA-5-HTT), melatonin, 5-methoxytryptamine (5-MTT), 3,4-dihydroxymandelic acid (DHMA), 5-hydroxytryptophol, and 5-methoxytryptophol (5-MTP), but not methanephine (MN), normethanephine (NMN), and 3-methyltyramine (3-MT), at 2 mM, inhibited para-aminohippuric acid uptake mediated by hOAT1. On the other hand, melatonin, 5-MI-3-AA, NA-5-HTT, 5-MTT, 5-MTP, HVA, 5-HI-3-AA, VMA, DOPAC, 5-hydroxytryptophol, and MN, but not 3-MT, DHMA, and NMN, at 2 mM, inhibited estrone sulfate uptake mediated by hOAT3. Differences in the IC₅₀ values between hOAT1 and hOAT3 were observed for DHMA, DOPAC, HVA, 5-HI-3-AA, melatonin, 5-MI-3-AA, 5-MTP, 5-MTT, and VMA. hOAT1 and hOAT3 mediated the transport of VMA but not HVA and melatonin. These results suggest that hOAT1 and hOAT3 are involved in the efflux of various neurotransmitter metabolites from the cerebrospinal fluid to the blood across the choroid plexus.

Keywords: organic anion transporter, choroid plexus, neurotransmitter metabolite, transport, efflux

Introduction

The choroid plexus found within the third, fourth, and lateral ventricles of the brain are formed from a single layer of epithelial cells surrounding the endothelium of blood capillaries (1). These polar epithelial cells are joined together by continuous tight junctions located near the apical side, which prevent significant movement of solutes from the blood into and out of the cerebrospinal fluid (2). The presence of efflux transport pathways for organic anions across the blood-cere-

brospinal fluid barrier was suggested in *in vivo* and *in vitro* kinetic studies. Organic anions, such as estradiol-17 β -glucuronide, benzylpenicillin, and cefodizime are actively transported from the cerebrospinal fluid into the circulating blood across the choroid plexus (3–6).

After their release from presynaptic neurons, certain amounts of neurotransmitters are metabolized by enzymes, including monoamine oxidase and catechol-*o*-methyltransferase. It remains unknown whether neurotransmitter metabolites induce neurotoxic effects, but it is possible that the accumulation of these metabolites affects the metabolism of neurotransmitters. In this regard, the efflux activity of neurotransmitter metabo-

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lites from the cerebrospinal fluid to the blood may be an important physiological function. In this regard, it was suggested that some neurotransmitter metabolites are transported via the organic anion transport system; 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) are actively transported from the cerebrospinal fluid into the blood by an energy-dependent, probenecid-sensitive transporter (7).

Recently, cDNAs encoding human organic anion transporters (hOATs) have been successively cloned, namely, hOAT1 (8, 9), hOAT2 (10), hOAT3 (11), and hOAT4 (12). On the other hand, cDNAs encoding another family of transporters mediating the transport of organic anions in rat have also been cloned, that is, organic anion-transporting polypeptide (oatp) 1, oatp2, and oatp3 (13–15). Northern blot analysis revealed that mRNAs for hOAT1, hOAT3, oatp1, oatp2, and oatp3 are expressed in the brain (9, 11, 13–15). In addition, rat OAT3 (rOAT3), oatp1, oatp2, and oatp3 were shown to be expressed in the choroid plexus (16–20). However, the expressions of hOAT1 and hOAT3 and their roles in the efflux of neurotransmitter metabolites in human choroid plexus have not been investigated yet.

The present study was undertaken to determine the localization of hOAT1 and hOAT3 in the human choroid plexus and to elucidate the interactions of hOAT1 and hOAT3 with various neurotransmitter metabolites using stable cell lines.

Materials and Methods

Materials

[¹⁴C]Para-aminohippuric acid (PAH) (53.1 mCi/mmol) and [³H]estrone sulfate (ES) (53 Ci/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA, USA). [¹⁴C]Melatonin (40 mCi/mmol) was purchased from Muromachi Chemicals Co. (Tokyo). Other materials used for medium preparation were described elsewhere (10, 21–23). Acid metabolites of neurotransmitters derived from catecholamines, namely, 3,4-dihydroxymandelic acid (DHMA), DOPAC, HVA, methanephine (MN), 3-methyltyramine (3-MT), normethanephine (NMN), and vanilmandelic acid (VMA), and those from serotonin, namely, 5-hydroxyindole-3-acetic acid (5-HI-3-AA), 5-hydroxytryptophol, melatonin, 5-methoxyindole-3-acetic acid (5-MI-3-AA), 5-methoxytryptamine (5-MTT), 5-methoxytryptophol (5-MTP), and *N*-acetyl-5-hydroxytryptamine (NA-5-HTT) were purchased from Sigma (St. Louis, MO, USA). Human adult normal choroid plexus tissue slides were purchased from Bio-chain Co., Ltd. (Hayward, CA, USA). Human choroid plexus tissues were obtained

from a 26 year-old male with informed consent from his family.

Immunohistochemistry

The generation of antibodies against hOAT1 and hOAT3 has been already described previously (9, 11). We have demonstrated that the cytoplasmic membranes of S₂ hOAT1 and S₂ hOAT3 were stained with polyclonal antibodies against hOAT1 and hOAT3, respectively (24). Light-microscopic analysis of the hOAT1 and hOAT3 was performed as previously described (24, 25). Briefly, the sections (2 μm) were cut and stained by the labeled streptavidin-biotin method. The sections were deparaffinized, incubated with 3% H₂O₂ for 15 min, and then incubated with blocking serum for 15 min. The sections were then incubated with polyclonal antibodies against hOAT1 (1:100 dilution) and hOAT3 (1:20 dilution) for 2 h. The sections were rinsed with Tris-buffered saline containing 0.1% Tween 20 and incubated with the biotinylated secondary antibody against rabbit immunoglobulin (Dako, Glostrup, Denmark) for 1 h. After rinsing with Tris-buffered saline containing 0.1% Tween 20, the sections were incubated for 30 min with horseradish peroxidase-conjugated streptavidin solution. Horseradish peroxidase labeling was detected using a peroxidase substrate solution with diaminobenzidine (0.8 mM; Dojindo Laboratories, Kumamoto). The sections were counterstained with hematoxylin before examination under a light microscope. As a negative control, an absorption test was performed with preincubation of the antibodies with epitopes of hOAT1 and hOAT3 (10 μg/ml each) for 2 h before applying the antibodies to the sections.

Cell culture

The establishment and characterization of the second segment of the proximal tubule (S₂) cells stably expressing hOAT1 and hOAT3 (S₂ hOAT1 and S₂ hOAT3) were performed as we previously described (10, 21–23). These cells were grown in a humidified incubator at 33°C under 5% CO₂ using the RITC 80-7 medium containing 5% fetal bovine serum, 10 mg/ml transferrin, 0.08 U/ml insulin, 10 ng/ml recombinant epidermal growth factor, and 400 μg/ml geneticin. The cells were subcultured in a medium containing 0.05% trypsin-EDTA solution (137 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 4 mM NaHCO₃, 0.5 mM EDTA, and 5 mM HEPES; pH 7.2) and used for 25–35 passages.

Inhibition study

Uptake experiments were performed as previously described (10, 21–23). S₂ hOAT1 and S₂ hOAT3 cells were seeded in 24-well tissue culture plates at a cell

density of 1×10^5 cells/well. After culture for two days, they were washed three times with Dulbecco's modified phosphate-buffered saline solution (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 , 1 mM KH_2PO_4 , 1 mM CaCl_2 , and 0.5 mM MgCl_2 ; pH 7.4), and then preincubated in the same solution in a water bath at 37°C for 10 min. S₂ hOAT1 and S₂ hOAT3 cells were incubated in a solution containing either 5 μM [^{14}C]PAH (hOAT1) or 50 nM [^3H]ES (hOAT3) in the presence or absence of various neurotransmitter metabolites at 37°C for 2 min. The reaction was stopped by the addition of ice-cold Dulbecco's modified phosphate-buffered saline, and the cells were washed three times with the same solution. The cells in each well were lysed with 0.5 ml of 0.1 N sodium hydroxide, and the radioactivity was determined using a scintillation counter (LSC-3100; Aloka, Tokyo). DHMA, 5-HI-3-AA, 5-MI-3-AA, 5-hydroxytryptophol, 5-MTP, 5-MTT, NA-5-HTT, and melatonin were dissolved in dimethylsulfoxide. Other substrates were dissolved in distilled water and then diluted with the incubation medium. The final concentration of dimethylsulfoxide in the incubation medium was adjusted to less than 1%.

Uptake of HVA, melatonin, and VMA

As described above, after culture for two days, they were washed three times with solution and then preincubated in the solution in a water bath at 37°C for 10 min. S₂ hOAT1, S₂ hOAT3, and mock cells were incubated in a solution containing either HVA at 0.5 μM for hOAT1 and 5 μM for hOAT3, [^{14}C]melatonin at 5 μM or VMA at 0.5 μM for hOAT1 and 10 μM for hOAT3 at 37°C for 5 min. The reaction was stopped by the addition of ice-cold Dulbecco's modified phosphate-buffered saline, and the cells were washed three times with the same solution. The amount of [^3H]melatonin within the cells was determined as described above. The intracellular concentrations of HVA and VMA were determined by HPLC as previously described (26). Briefly, a high-performance liquid chromatograph was equipped with a L-6200 pump (Hitachi Ltd., Tokyo). The samples were injected through a 20- μl loop injector. Effluent fluorescence was detected with a spectrofluorometer (FP-821; Nihon Bunko, Tokyo), with a 285-nm excitation wavelength and a 320-nm emission wavelength. The detector was connected to a recorder-integrator (LC-5A; Shimadzu, Kyoto). The mobile phase consisted of potassium phosphate buffer (20 mM, pH 4.0), combined with methanol (HPLC grade; Merck, Darmstadt, Germany) by a gradient-elution technique at a flow-rate of 1.1 ml/min.

Statistical analyses

Data are expressed as the mean $\pm$ S.E.M. Statistical differences were determined using one-way analysis of variance with Dunnett's post-hoc test for the inhibition and VMA uptake experiments and the unpaired Student's *t*-test for the comparison of IC₅₀ values. When the IC₅₀ values were more than 2 mM, the comparison was made considering the IC₅₀ values as 2 mM. Differences were considered significant at $P < 0.05$.

Results

hOAT1 and hOAT3 expression in the choroid plexus of human brain

Figure 1 shows immunohistochemical analysis of hOAT1 and hOAT3 in the human choroid plexus. The cytoplasmic membrane and cytoplasm of the choroid plexus showed positive immunoreactivities with the antibodies against hOAT1 (Fig. 1A) and hOAT3 (Fig. 1B). The specificity of the reaction for hOAT1 and hOAT3 in the choroid plexus was demonstrated by a reabsorption test performed with hOAT1 (Fig. 1C) and hOAT3 (Fig. 1D) that showed no staining.

Interactions of hOAT1 and hOAT3 with neurotransmitter metabolites

We have examined the effects of various neurotransmitter metabolites on the organic anion uptake mediated by hOAT1 and hOAT3. As shown in Fig. 2A, 5-MI-3-AA, HVA, VMA, DOPAC, 5-HI-3-AA, NA-5-HTT, melatonin, 5-MTT, DHMA, 5-hydroxytryptophol, and 5-MTP, but not MN, NMN, and 3-MT, inhibited PAH uptake mediated by hOAT1 ($N = 6$, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs control). On the other hand, as shown in Fig. 2B, melatonin, 5-MI-3-AA, NA-5-HTT, 5-MTT, 5-MTP, HVA, 5-HI-3-AA, VMA, DOPAC, 5-hydroxytryptophol, and MN, but not 3-MT, DHMA and NMN, inhibited ES uptake mediated by hOAT3 ($N = 6$, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs control). We also examined the effects of various concentrations of these neurotransmitter metabolites on organic anion uptake mediated by hOAT1 and hOAT3. As shown in Fig. 3, HVA inhibited organic anion uptake mediated by hOAT1 (Fig. 3A) and hOAT3 (Fig. 3B) in a dose-dependent manner ($N = 6$, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs control). Table 1 shows the IC₅₀ values of catecholamine metabolites for the organic anion uptake mediated by hOAT1 and hOAT3, and Table 2 shows those of serotonin metabolites for hOAT1 and hOAT3.

Uptake of HVA, melatonin, and VMA by hOAT1 and hOAT3

We have elucidated whether hOAT1 and hOAT3

Fig. 1. Immunohistochemistry for hOAT1 and hOAT3 in choroid plexus of human brain. Epithelium of human choroid plexus was stained with polyclonal antibodies against hOAT1 (A) and hOAT3 (B). As a negative control, the reabsorption test was performed for hOAT1 (C) and hOAT3 (D). The bar indicates 50 μm .

mediate the uptake of HVA, melatonin, and VMA. As shown in Table 3, hOAT1 and hOAT3 exhibited a higher uptake activity of VMA, but not HVA and melatonin, than the mock cells, indicating that hOAT1 and hOAT3 mediate the transport of VMA ($N = 6$, $**P < 0.01$ and $***P < 0.001$ vs mock cells).

Discussion

HOAT1 and hOAT3 have been shown to mediate the transport of nonsteroidal anti-inflammatory drugs, antitumor drugs, histamine H_2 -receptor antagonists, prostaglandins, diuretics, angiotensin-converting enzyme inhibitors, and beta-lactam antibiotics (9, 11, 21–23). Some differences in characteristics exist between hOAT1 and hOAT3, such as substrate specificity and localization (9, 11). In the current study, we elucidated the expression of hOAT1 and hOAT3 in the choroid plexus of the human brain and their interactions with various neurotransmitter metabolites using stable cell lines.

HOAT1 and hOAT3 are expressed in the cytoplasmic

membrane and the cytoplasm of human choroid plexus. ROAT3, but not rOAT1, was shown to be expressed in the brush border membrane of the choroid plexus epithelial cells in rats (16). Although the polarized localization of hOAT1 and hOAT3 remains unclear, there appears to be an interspecies difference in the expression of OAT1 between humans and rats. It is possible that these transporters are involved in the efflux of anionic compounds from the cerebrospinal fluid to the blood based on the following reasons: 1) It was reported that hOAT1 exhibits transport property as an exchanger (9); 2) rOAT3 exhibits functions as an organic anion/dicarboxylate exchanger (27), and we have also demonstrated that hOAT3 mediates the uptake as well as the efflux of tetracycline (23); 3) In rats, it was suggested that rOAT3 functions in one of the mechanisms for the removal of organic anions from the cerebrospinal fluid (16).

The neurotransmitter metabolites tested inhibited the organic anion uptake mediated by hOAT1 and hOAT3. The rank order of the apparent IC_{50} values for

Fig. 2. Effects of neurotransmitter metabolites on organic anion uptake mediated by hOAT1 (A) and hOAT3 (B). S₂ hOAT1 and S₂ hOAT3 cells were incubated in a solution containing either 5 μ M [¹⁴C]PAH (hOAT1) or 50 nM [³H]ES (hOAT3) for 2 min in the absence or presence of 2 mM neurotransmitter metabolites. Each data represents the mean $\pm$ S.E.M. of six monolayers from two separate experiments. * P <0.05, ** P <0.01, and *** P <0.001 vs control.

hOAT1 was 5-MI-3-AA < HVA < VMA < 5-HI-3-AA < NA-5-HTT < DOPAC < melatonin < DHMA < 5-MTT, while that for hOAT3 was melatonin < 5-MI-3-AA < NA-5-HTT = 5-MTP < 5-MTT < HVA < 5-HI-3-AA < DOPAC < VMA. In addition, IC₅₀ values of DHMA, DOPAC, HVA, 5-HI-3-AA, 5-MI-3-AA, and VMA for hOAT1 were significantly lower than those for hOAT3, whereas those of melatonin, 5-MTT, and 5-MTP for hOAT3 were significantly lower than those for hOAT1.

Fig. 3. Effects of various concentrations of HVA on organic anion uptake mediated by hOAT1 and hOAT3. S₂ hOAT1 and S₂ hOAT3 cells were incubated in a solution containing either 5 μ M [¹⁴C]PAH (hOAT1) or 50 nM [³H]ES (hOAT3) for 2 min in the absence or presence of various concentrations of HVA. Each data represents the mean $\pm$ S.E.M. of six monolayers from two separate experiments. * P <0.05, ** P <0.01, and *** P <0.001 vs control.

Thus, it appears that there exists a significant difference in substrate recognition between hOAT1 and hOAT3 for neurotransmitter metabolites. We have already demonstrated that 5-MI-3AA, 5-MTP, melatonin, and 5-HI-3-AA at 1 mM inhibited ES uptake mediated by rOAT3 expressed in *Xenopus laevis* oocytes, whereas DHMA inhibited it very weakly (28).

Among the neurotransmitter metabolites tested, DHMA, DOPAC, VMA, HVA, 5-HI-3-AA, and 5-MI-3-AA possess carboxylic groups. The IC₅₀ values of these substrates for hOAT1 were lower than those for hOAT3. The results suggest that the existence of the

Table 1. IC₅₀ values of catecholamine metabolites for the organic anion uptake mediated by hOAT1 and hOAT3

Compounds	hOAT1	hOAT3
	(mM)	
DHMA	1.09 ± 0.21***	2>
DOPAC	0.56 ± 0.09*	0.99 ± 0.10
HVA	0.065 ± 0.006***	0.76 ± 0.09
MN	2>	2>
3-MT	2>	2>
NMN	2>	2>
VMA	0.07 ± 0.01***	1.24 ± 0.40

S₂ hOAT1 and S₂ hOAT3 cells were incubated in a solution containing either 5 μM [¹⁴C]PAH (hOAT1) or 50 nM [³H]ES (hOAT3) at 37°C for 2 min in the absence or presence of various catecholamine metabolites. Each data represents the mean ± S.E.M. of six monolayers from two separate experiments. When the IC₅₀ value was more than 2 mM, statistical analysis was performed considering that it is 2 mM. *P<0.05 and ***P<0.001 vs hOAT3.

Table 2. IC₅₀ values of serotonin metabolites for the organic anion uptake mediated by hOAT1 and hOAT3

Compounds	hOAT1	hOAT3
	(mM)	
5-Hydroxytryptophol	2>	2>
5-HI-3-AA	0.11 ± 0.02***	0.91 ± 0.03
Melatonin	0.91 ± 0.03***	0.05 ± 0.01
5-MI-3-AA	0.03 ± 0.001*	0.07 ± 0.01
5-MTP	2>***	0.49 ± 0.10
5-MTT	1.38 ± 0.16**	0.61 ± 0.16
NA-5-HTT	0.44 ± 0.09	0.49 ± 0.06

S₂ hOAT1 and S₂ hOAT3 cells were incubated in a solution containing either 5 μM [¹⁴C]PAH (hOAT1) or 50 nM [³H]ES (hOAT3) at 37°C for 2 min in the absence or presence of various serotonin metabolites. Each data represents the mean ± S.E.M. of six monolayers from two separate experiments. When the IC₅₀ value was more than 2 mM, statistical analysis was performed considering that it is 2 mM. *P<0.05, **P<0.01, and ***P<0.001 vs hOAT3.

Table 3. HVA, melatonin, and VMA uptake by hOAT1 and hOAT3

Compounds	hOAT1	hOAT3	Mock
	(pmol·mg protein ⁻¹ ·5 min ⁻¹)		
HVA	73.8<	47.8<	31.6<
Melatonin	16.8 ± 1.23	29.8 ± 7.32	19.3 ± 4.18
VMA	118 ± 8.19***	62.8 ± 14.0**	29.0<

S₂ hOAT1, S₂ hOAT3, and mock cells were incubated in a solution containing either HVA at 0.5 μM for hOAT1 and 5 μM for hOAT3, 5 μM [¹⁴C]melatonin or VMA at 0.5 μM for hOAT1 and 10 μM for hOAT3 at 37°C for 5 min. The intracellular contents of VMA and HVA were determined by HPLC. Each data represents the mean ± S.E.M. of six monolayers from two separate experiments. **P<0.01 and ***P<0.001 vs mock cells. Statistical analysis of VMA uptake was performed as the amount of uptake by mock cells was 29.0 pmol·mg protein⁻¹·5 min⁻¹.

negative charge in the substrates increase their affinities for hOAT1 rather than for hOAT3. This tendency was consistent with a previous report concerning the interaction of nonsteroidal anti-inflammatory drugs with rOAT1 expressed in *Xenopus laevis* oocytes (29).

The current results showed that hOAT1 and hOAT3 interact with various neurotransmitter metabolites. In addition, we found that hOAT1 and hOAT3 mediate the transport of VMA but not HVA and melatonin. The transport of substrates consists of three processes, that is, substrate binding, translocation, and dissociation. Thus, the inhibitory effects of neurotransmitter metabolites other than VMA, HVA, and melatonin on hOAT1 and hOAT3 do not necessarily indicate that these substrates are transported by hOAT1 and hOAT3. In order to establish whether these neurotransmitter metabolites are indeed effluxed from the cerebrospinal fluid to the blood across the choroid plexus, experiments on the uptake or the efflux of these substrates by hOAT1 and hOAT3 should be performed.

We have previously demonstrated that hOAT1 and hOAT3 mediate the transport of clinically used drugs including nonsteroidal anti-inflammatory drugs such as salicylate, ketoprofen, ibuprofen, and indomethacin, as well as antiviral agents including 3'-azido-3'-deoxythymidine and acyclovir (21, 22). These drugs have been shown to induce various forms of neurological adverse drug reactions (30–32). In this regard, it is possible that hOAT1 and hOAT3 are involved in the efflux of these drugs across the blood-cerebrospinal fluid barrier and are associated with the induction of neurological adverse drug reactions.

In conclusion, it was suggested that hOAT1 and hOAT3 are involved in the efflux of various neurotransmitter metabolites from the cerebrospinal fluid to the blood across the choroid plexus.

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