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Inhibitory and facilitory actions of isocyanine derivatives at human and rat organic cation transporters 1, 2 and 3: A comparison to human α_1- and α_2-adrenoceptor subtypes

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ABSTRACT

Organic cation transporters (OCTs), comprising OCT1, OCT2 and OCT3 subtypes, control absorption and elimination of xenobiotics and endogenous compounds in kidney, liver and placenta. In addition, they ensure "uptake$_2$", low-affinity catecholamine clearance in sympathetically-innervated tissue and the CNS. The prototypical OCT ligand, disprocynium24 (D24), recognises OCT3, but its actions at OCT1 and OCT2 remain unknown. Herein, together with two other isocyanine derivatives (AAC291 and AAC301) and chemically-related adrenergic agents, we evaluated actions of D24 at OCTs, monoamine transporters and α_1- and α_2-adrenoceptors. D24 concentration-dependently suppressed [^{3}H]-1-methyl-4-phenylpyridinium (MPP^+) transport at human (h) and rat (r) OCT1, OCT2 and OCT3 in stably transfected HEK293 cells. Interestingly, low concentrations of D24 *enhanced* transport by h/rOCT2, a substrate-dependent effect suppressed by inhibition of protein kinase C. AAC291 and AAC301 likewise inhibited transport by all classes of h/r OCT and at low concentrations induced even more marked increases in transport by h/rOCT2. Further, by analogy to D24, they displayed antagonist properties at h$\alpha_{1A/B/D}$-adrenoceptors (Ca^{2+}-flux) and h$\alpha_{2A/B/C}$-adrenoceptors ([^{35}S]GTPγS binding). They were, however, less potent than D24 at serotonin transporters ([^{3}H]citalopram binding) and AAC291 did not bind to dopamine and norepinephrine transporters. The preferential α_{1B}-adrenoceptor antagonist, AH11110A, the α_2-adrenoceptor agonist, RWJ52353, and the adrenergic neurotoxin DSP-4 likewise affected [^{3}H]MPP^+ transport, in an OCT-subtype and species-dependent manner. In conclusion, D24, other isocyanine congeners and chemically-related adrenergic agents inhibit OCT-mediated [^{3}H]MPP^+ transport, and all drugs display significant activity at α_1- and α_2-adrenoceptor subtypes, expanding previous reports of promiscuity between pharmacophores recognising α-adrenoceptors and OCTs.

1. Introduction

Organic cation transporters (OCTs) belong to the Slc22 family of membrane transporters. They play important roles in controlling the bioavailability and detoxification of a wide range of endogenous substances and xenobiotics in peripheral organs (Koepsell et al., 2007). The three major OCT subtypes, OCT1, OCT2 and OCT3, accept a variety of substrates generally comprising small, positively charged organic cations but also in some cases neutral compounds (Koepsell et al., 2007). Drugs transported by this category of transporter include antimalarials like quinine, antidiabetics like metformin, various antiviral agents and cytostatics and several histamine H_2 receptor antagonists, such as cimetidine (Koepsell et al., 2007). In agreement with their distribution and localization in kidney, liver, intestine and placenta, OCTs tightly control the balance between absorption and excretion of their substrates. In addition, these transporters ensure uptake$_2$, a low-affinity catecholamine clearance system present in sympathetically-innervated organs and tissues, which is also called "extraneuronal monoamine transport" (Iversen, 1965; Trendelenburg, 1988). The role of this system was particularly well illustrated for cardiac muscle (Iversen, 1965), and smooth muscle (Trendelenburg, 1988) in early studies

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of catecholamine uptake in isolated perfused organs or infused animals.

The importance of the overall contribution of OCTs to the handling of organic cations in peripheral organs was confirmed by studies of mouse mutants deficient for one or several of these transporters (Jonker et al., 2001, 2003; Zwart et al., 2001). Furthermore, OCT3 was shown to be essential for the homeostatic regulation of aminergic neurotransmission in the brain (Millan, 2006). Mice deficient for OCT3 show changes of the neural and behavioural responses to environmentally-induced variations in osmolarity (Vialou et al., 2004), altered monoamine neurotransmission in the brain, increased sensitivity to psychostimulants and decreased sensitivity to dopaminergic neurotoxicity induced by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Cui et al., 2009; Vialou et al., 2008). This «back-up» role in monoamine clearance is supported by studies of mice injected with OCT3-antisense oligonucleotides (Nakayama et al., 2007) or lacking serotonin (5-HT) transporters (Baganz et al., 2008; Daws, 2009).

The above observations suggest that OCTs participate in various physiological functions, and that alterations in their activity may influence drug absorption and clearance, as well as aminergic neurotransmission in the central and peripheral nervous systems. OCT activity is regulated both at the transcriptional level (Asaka et al., 2006) and by phosphorylation (see Discussion), but the best characterised mechanism of modulation of OCT activity is via direct action of inhibitors. These include OCT cognate substrates themselves, many (often larger) organic cations, organic anions and neutral compounds such as corticosteroids, ion channel blockers, neurotransmitter receptor ligands, psychostimulants and antidepressants (Koepsell et al., 2007).

Interestingly, relatively potent inhibition is exerted by certain α-adrenoceptor ligands, such as the α_1-adrenoceptor antagonists phenoxybenzamine and prazosin and the α_2-adrenoceptor partial agonist clonidine (Hayer-Zillgen et al., 2002). Furthermore, earlier studies of extraneuronal catecholamine transport in an *ex vivo* model, Caki-1 cells, led to identification of high-affinity OCT inhibitors, decynium22 (1,1′-diethyl-2,2′-cyanine) and the structurally-related disprocynium24 (D24, 1,1′-diisopropyl-2,4-cyanine). These compounds bind native rodent α_1- and α_2-adrenoceptors (Russ et al., 1993, 1996), but their actions at human α_1- and α_2-adrenoceptor subtypes remain to be characterised. D24 and decynium22 have proven invaluable in establishing OCT identity with hepatic and renal organic cation transport systems (Grundemann et al., 1998; Zhang et al., 1997) and with "extraneuronal monoamine transport" (Eisenhofer et al., 1996; Graefe et al., 1997; Grundemann et al., 1997). Further, with the exception of the very recently-described OCT ligand lamivudine (a nucleoside reverse transcriptase inhibitor) (Minuesa et al., 2009), they remain the most potent and specific OCT inhibitors identified to date (Russ et al., 1996). Notably, D24 has proven a particularly effective inhibitor of extraneuronal transport (Russ et al., 1993) and OCT3 (Grundemann et al., 1998, 2002), but its efficacy at OCT1 and OCT2 has not as yet been determined.

In light of the above observations, the present studies: *first*, investigated the effects of D24 and two structurally-related analogues, AAC291 and AAC301 (Fig. 1), on [^{3}H]MPP$^+$ transport in HEK293 cells transfected with human or rat OCT1, OCT2 or OCT3. *Second*, the actions of DSP-4, an adrenergic neurotoxin that shares the quaternary ammonium structure of D24 (Lyons et al., 1989), were investigated. Further, we also examined the effects of the α_2-adrenoceptor agonist, RWJ52353 (Ross et al., 2000) and of the selective α_{1B}-adrenoceptor antagonist, AH11110A, since they likewise possess basic nitrogens protonable at physiological pH (Assari et al., 2003; Eltze et al., 2001; Giardina et al., 1996) (Fig. 1). *Third*, the affinities of all drugs were determined at human 5-HT, norepinephrine and dopamine transporters, and we also determined both affinities and functional actions of drugs at multiple subtypes of cloned hα_1-adrenoceptors and hα_2-adrenoceptors. *Finally*, we extended our studies to an initial exploration of potential cellular mechanisms underlying the activation of hOCT2-mediated transport by low concentrations of D24.

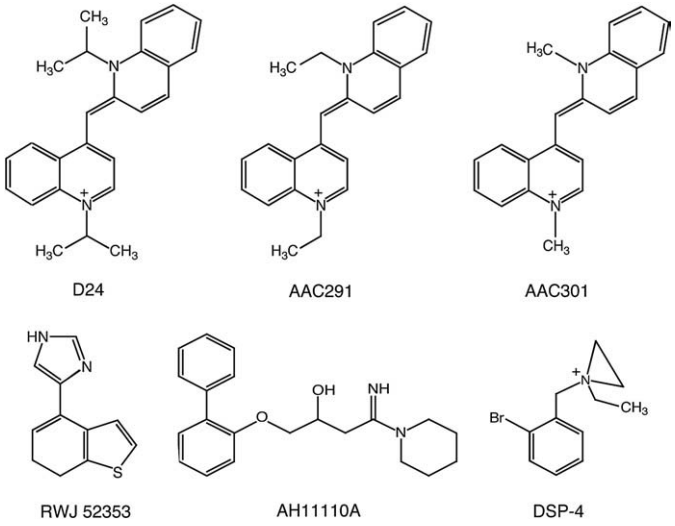

Fig. 1. Chemical structures of drugs studied for their influence upon [^{3}H]MPP$^+$ transport in HEK293 cells stably transfected with human (h) or rat (r) organic cation transporters (OCTs). Disprocynium24 (D24), AAC291 and AAC301 are isocyanine derivatives; AH11110A is a preferential hα_{1B}-adrenoceptor antagonist (see Table 1), RWJ52353, an α_2-adrenoceptor agonist (see Table 1) and DSP-4, which shares the quaternary structure of D24, is an adrenergic neurotoxin.

2. Materials and methods

2.1. Drugs and substances

[^{3}H]-1-methyl-4-phenylpyridinium acetate ([^{3}H]MPP$^+$) (3.09 Tbq/mmol), and [^{35}S]GTPγS (37 Tbq/mmol) were obtained from PerkinElmer (Life Sciences, Inc., Boston) and [^{3}H]5-HT (570 Gbq/mmol) from Amersham (Amersham Biosciences, Buckinghamshire, UK). 1,1′-diisopropyl-2,4-cyanine iodide (disprocynium24), 1,1′-diethyl-2,4-cyanine iodide (AAC 291) and 1,1′-dimethyl-2,4-cyanine iodide (AAC 301) were synthesized by Servier Laboratories (AC). These disprocynium derivatives, fully verified by NMR studies, are isomers of structures disclosed in the original papers on these compounds (Russ et al., 1993). 1-(Biphenyl-2-yloxy)-4-imino-4-piperidin-1-ylbutan-2-ol (AH11110A), *4-(6,7*-Dihydro-benzo (b)thiophen-4-yl)-1H-imidazole (RWJ52353) and N-(2-Chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) were also synthesized by Servier. Ro 32-0432, KT 5720, and KN 93 were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.2. Cell culture

Stably transfected HEK293 (ATCC-CRL-1573) cells expressing human (Hayer-Zillgen et al., 2002) or rat (Amphoux et al., 2006) OCT1, OCT2 or OCT3 have been described previously. HEK293 cells were used to generate stably transfectants in view of the very low expression of endogenous OCTs in this cell line, as demonstrated by the absence of significant uptake of [3H]MPP$^+$ in non transfected cells. All cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Gaithersburg, MD) at 37 °C in a humidified atmosphere (5% CO_2). The cells were subcultured every 5 days to maintain exponential growth, and medium was replaced every 2 to 3 days.

2.3. Transport assays

Stably transfected HEK293 cells expressing human or rat OCTs were plated at the density of 5.10^4 cell/well in 24-well plates coated with 0.1 g/l poly-L-ornithine (Sigma-Aldrich, St Louis, MO, USA) and grown to subconfluence. Cells were preincubated for 15 min at 37 °C in a Krebs Ringer Hepes (KRH) buffer (25 mM HEPES–NaOH, pH 7.4, 125 mM NaCl, 4.8 mM KCl, 5.6 mM D(+)-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4 and 1.2 mM $MgSO_4$) then incubated for 15 min at 37 °C in the same buffer containing 25 nM [^{3}H]MPP^+ or 100 nM [^{3}H] 5-HT as described (Amphoux et al., 2006). The cells were rinsed 3 times with ice-cold KRH buffer and the proteins were solubilized with 0.1 M NaOH. Radioactivity was then assessed by liquid scintillation counting and protein concentration was measured by the method of Bradford standardized with bovine serum albumine in order to normalize uptake values. Specificity of MPP^+ uptake was verified by inhibition with D24 (10^{-4}M). Uptake of 25 nM ^{3}H-MPP^+ under control conditions was: 30 ± 2.5 fmol mg^{-1} min^{-1} for rOCT1, 30 ± 3 fmol mg^{-1} min^{-1} for rOCT2, 60 ± 8.7 fmol mg^{-1} min^{-1} for rOCT3, 30 ± 9.2 fmol mg^{-1} min^{-1} for hOCT1, 40 ± 4.9 fmol mg^{-1} min^{-1} for hOCT2 and 60 ± 8.3 fmol mg^{-1} min^{-1} for hOCT3, respectively. For transport inhibition experiments, increasing concentrations of drugs were added during pre-incubation and maintained during uptake. To determine whether D24 interacted irreversibly with hOCT2, washing steps in KRH buffer (3×5 min) were added after pre-incubation. To evaluate the potential involvement of protein kinase pathways in the activation of hOCT2 by low concentrations of D24, specific inhibitors of protein kinase A, protein kinase C and calmodulin kinase II (CaMKII), KT 5720 (Hidaka et al., 1984), Ro 32-0432 (Wilkinson et al., 1993) and KN 93 (Mamiya et al., 1993) respectively, were added during the pre-incubation and incubation period.

2.4. Data analysis of drug modulation of [^{3}H]MPP^+ transport

The effect of various compounds on OCT transport was determined by non-linear regression analysis with a one site binding model for standard inhibition and a bell-shaped model in case of activation (GraphPad Prism software, San Diego, CA, USA). For standard inhibition, non-saturating concentrations of [^{3}H]MPP^+ (25 nM) were used, much lower than K_m values for MPP^+ uptake by OCTs (10 to 150 μM). IC_{50} values were calculated as arithmetic means of up to 5 experiments ± S.E.M.

2.5. Membrane preparations

Frontal cortices from male Wistar rats (225–300 g) (Charles River, L'Arbresle, France) were thawed and homogenized using a Polytron in 20 volumes of ice-cold assay buffer containing Tris–HCl (50 mM, pH 7.6), $CaCl_2$ (4 mM), ascorbic acid (0.1% w/v) and pargyline (10 μM) and centrifuged (20 min, 35,000*g*) at 4 °C. The supernatant was discarded and pellets were resuspended in 20 volumes of ice-cold assay buffer. The homogenate was incubated 15 min at 37 °C to remove endogenous ligand and then recentrifuged (20 min, 35,000*g*). Finally, the resulting pellets were resuspended in the same buffer (1/300, w/v) and used for determination of drug affinities at α1A and α2D receptors.

2.6. Determination of drug affinities at monoamine transporters and subtypes of hα$_1$ and hα$_2$ adrenergic receptors

Methods employed either on rat cortex membrane preparation or on stably transfected cell membrane preparation (summarized in Table 1) were described previously (Millan et al., 2000, 2004). Isotherms were analysed by non-linear regression using PRISM (GraphPad Software, San Diego, CA) to generate inhibitory concentration (IC) values. They were transformed into K_i values according to Cheng-Prussof (Cheng and Prusoff, 1973): K_i = inhibitory concentration$_{50}$/(1 + *L*/Kd), where *L* is the radioligand concentration and Kd is the dissociation constant, and are presented as pK_i values (negative log10 of K_i).

2.7. Agonist and antagonist properties at subtypes of hα$_2$-adrenoceptors: guanosine-5′0-(3-[35S]thio)-triphosphate ([^{35}S]GTPγS) binding

The procedures used for determination of the functional action of compounds at hα$_{2A}$, hα$_{2B}$ and hα$_{2C}$-adrenoceptors expressed in Chinese Hamster Ovary (CHO) cells by [^{35}S]GTPγS (1000 Ci/mmol, PerkinElmer Life Sciences, Boston, MA) binding have been described in detail elsewhere (Millan et al., 2000, 2004; Newman-Tancredi et al., 1998). Briefly, [^{35}S]GTPγS was employed at a concentration of 0.2 nM. In each case, the pH was 7.4, the temperature was 22 °C and the incubation period was 40 min. The buffer comprised HEPES (20 mM), NaCl (100 mM), guanosine diphosphate (GDP) (3 μM) and $MgCl_2$ (3 mM). For evaluation of the agonist actions, membranes were incubated with incremental concentrations of compounds. For evaluation of the antagonist properties of compounds, interaction studies were undertaken with a fixed concentration of norepinephrine (10 μM). Experiments were terminated by rapid filtration through Unifilter-96 GF/B filters using a filtermate harvester (PerkinElmer). The radioactivity retained on the filters was quantified by scintillation counting. K_B values for inhibition of norepinephrine-stimulated [^{35}S]GTPγS binding by compounds were calculated according to Cheng–Prusoff: $K_B = IC_{50}/(1 + (\text{agonist}/EC_{50}))$, where IC_{50} = inhibitory concentration$_{50}$ of compound, agonist = concentration of norepinephrine and EC_{50} = effective concentration$_{50}$ of norepinephrine alone.

2.8. Agonist and antagonist properties at hα$_{1A}$-adrenoceptors: measurement of intracellular Ca^{2+} concentrations by fluorometry

CHO cells stably expressing the human recombinant adrenoceptors (cell lines established in-house) were seeded 24 h before assay at 40 000 cells per well into 96-well black-walled culture plates coated with poly-D-lysine. Cells were loaded with a calcium kit assay buffer (Molecular Devices) containing 2.5 mM probenecid and incubated at 37 °C for 1 h in 6% CO_2 atmosphere. After 10 s, the agonist was added. For antagonist studies, the compounds to be tested were added 10 min before the addition of norepinephrine. The increase of intracellular Ca^{2+} was monitored using the FLIPR detection system (Fluorometric Imaging Plate Reader, Molecular Devices) at 488 nm for 120 s. Agonist efficacy (E_{max}) is expressed relative to that of norepinephrine (=100%) which was tested at a maximally effective concentration of 1 μM in each experiment. Antagonist potencies are expressed as IC_{50}, where IC_{50} is the inhibitory concentration of antagonist that gives 50% inhibition of calcium flux in the presence of a fixed concentration of agonist ([ago]) and EC_{50}ago is the EC_{50} of the agonist when tested alone.

3. Results

3.1. Comparative influence of D24 and related isocyanine analogues upon OCT-mediated [^{3}H]MPP^+ uptake in stably transfected HEK293 cells

When increasing concentrations of D24 or the two other cyanine derivatives (Fig. 1) were added during pre-incubation and uptake, two different types of effect upon [^{3}H]MPP^+ uptake by human and rat OCTs were observed (Fig. 2). An inhibitory effect with a standard sigmoid curve was found with all 3 isocyanines on hOCT3, as well as with D24 on rOCT1 and hOCT1. In the other instances, more complex bell-shaped profiles were seen, with transport initially enhanced by *low* concentrations (in the 0.1–1 μM range) of isocyanines followed by inhibition at higher concentrations. In terms of inhibition, D24 was the most potent drug, with inhibitory constants (IC_{50} values) of 400 nM, 2.8 μM and

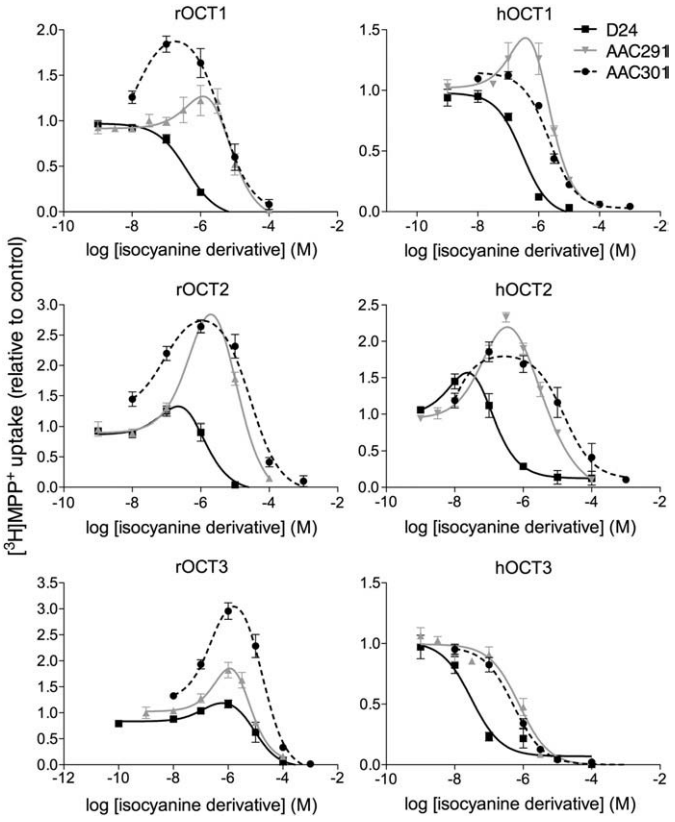

Fig. 2. Influence of isocyanine derivatives upon [^{3}H]MPP$^+$ uptake in HEK293 cells stably transfected with human (h) or rat (r) OCTs. Uptake of [^{3}H]MPP$^+$ (25 nM) was evaluated for 15 min at 37 °C in the presence of increasing concentrations of D24 (■), its ethyl analogue, AAC291(▲) or its methyl analogue, AAC301 (●) during pre-incubation and incubation. Data are expressed as arithmetic means ± S.E.M. of control uptake (in the absence of inhibitors) values for up to 5 independent experiments performed in triplicate.

17.8 µM for rOCT1, rOCT2 and rOCT3, and 306 nM, 280 nM and 31 nM, for hOCT1, hOCT2 and hOCT3, respectively (Fig. 2). The ethyl and methyl isocyanine derivatives, AAC291 and AAC301, were less potent inhibitors compared to D24, yielding values of respectively 2.26 µM for AAC301 at hOCT1 and about 0.5–0.7 µM for both compounds at hOCT3 (Fig. 2). For the rat OCT subtypes, IC_{50} values for AAC291 and AAC301 were 14 and 16 µM for rOCT1, 40 and 126 µM for rOCT2 and 22.4 and 89 µM for rOCT3, respectively. The inhibition constant determined here for D24 at hOCT3 is consistent with that reported for hOCT3-transfected cells (Grundemann et al., 1998), and for norepinephrine uptake by Caki cells, a model system for OCT3 study.

For hOCT2, a maximum activation of [^{3}H]MPP$^+$ uptake was achieved at approximately 10 nM of D24, whereas higher concentrations (approximately 1 µM) of AAC291 and AAC301 were needed for activation (Fig. 2). Interestingly, with these two D24 analogues, the activating effect was not only displaced toward higher concentrations, but also significantly (2-fold) stronger than with D24 itself. Hence, in the 0.1 µM range, these two isocyanines selectively activated hOCT2 without affecting hOCT1 or hOCT3. For rOCT subtypes as well, an activation peak was induced by the 3 cyanine compounds at concentrations of approximately 1 µM (Fig. 2). Again, the intensity of this activation was noticeably stronger (3 to 5 fold) for AAC291 and AAC301 as compared with D24.

Thus, the isocyanine analogues AAC 291 and AAC 301 were less potent inhibitors than D24 itself but exhibited (at higher concentrations) stronger activating properties. Overall, these data confirm the interaction of isocyanine-like structures with all OCT subtypes.

3.2. Influence of the α-adrenoceptor ligands AH11110A and RWJ52353 upon OCT-mediated [^{3}H]MPP$^+$ uptake in stably transfected HEK293 cells

Based on the observation that isocyanine-related OCT inhibitors recognise α_1- and α_2-adrenoceptors (Russ et al., 1993), we explored the effect on OCT transport of the preferential α_{1B}-adrenoceptor antagonist, AH111101A (Assari et al., 2003; Eltze et al., 2001; Giardina et al., 1996) and the α_2-adrenoceptor agonist, RWJ52353 (Ross et al., 2000) (Fig. 1). At first glance, the structural similarity between these α-adrenoceptor ligands and D24 is not evident, but these (and certain other) agents possess basic nitrogen atom which can be protonated at physiological pH, mimicking the quaternary ammonium function present in disprocynium. AH11110A showed comparable inhibitory effects on rat and human OCT1 subtypes, with IC_{50} values of 12.6 and 2.5 µM, respectively (Fig. 3). Similar effects were likewise apparent for rOCT2 and hOCT2 with a marked activation peak in both cases in the 0.1 µM to 10 µM range and inhibition seen at higher concentrations with IC_{50}s of 112 and 63 µM, respectively. Only for OCT3 did the action of the compound differ between species, with a facilitation peak for rOCT3 but not its human counterpart, and inhibition with IC_{50}s of 316 and 11.2 µM, respectively (Fig. 3).

The α_2-adrenoceptor agonist RWJ52353 exerted an inhibitory effect on rOCT1 and rOCT2 at relatively high concentrations (IC_{50} 100 and 20 µM, respectively), whereas it strongly activated rOCT3 (3-fold). For this subtype, as well as for the 3 human subtypes, IC_{50}s were above the 100 µM range (Fig. 3). At concentrations lower than those causing inhibition, an activation peak was also seen with this agent in

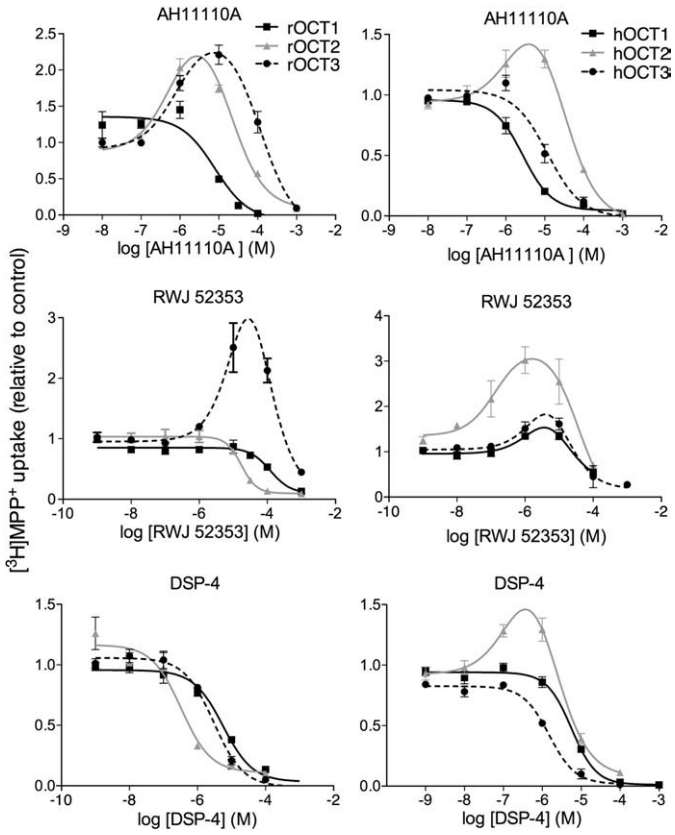

Fig. 3. Influence of adrenergic ligands upon [^{3}H]MPP$^+$ uptake in HEK293 cells stably transfected with human (h) or rat (r) OCTs. Uptake of [^{3}H]MPP$^+$ (25 nM) was evaluated for 15 min at 37 °C in the presence of increasing concentrations of AH11110A, a preferential hα_{1B}-adrenoceptor antagonist; RWJ52353, an α_2-adrenoceptor agonist and DSP-4, an adrenergic neurotoxin. Data for rat and human OCT1 (■), OCT2 (▲) and OCT3 (●) mediated transport is expressed as the arithmetic means ± S.E.M. of control uptake (in the absence of inhibitors) values for up to 5 independent experiments performed in triplicate.

the 1–10 μM range for all human OCT subtypes, though it was more pronounced for hOCT2.

3.3. Influence of the adrenergic neurotoxin, DSP-4, upon OCT-mediated [^{3}H-MPP$^+$] uptake in stably transfected HEK293 cells

DSP-4 is a neurotoxin chemically related to D24 which selectively lesions noradrenergic neurons emanating from the locus coeruleus (Lyons et al., 1989). While the norepinephrine transporter (NET) has been assumed to be its major target, *via* which it enters cells (Grzanna et al., 1989), this has never actually been shown, and other sites of action have also been suspected (Lyles and Callingham, 1981). DSP-4 robustly suppressed transport at all 3 classes of rat OCT and, in distinction to other ligands examined, displayed in most cases a straightforward inhibitory effect, with IC_{50}s of 6.3 and 5 μM for rOCT1 and hOCT1, 0.5 μM for rOCT2 and 3.2 and 1.6 μM for rOCT3 and hOCT3, respectively (Fig. 3). Only at hOCT2 was an activating component observed, with DSP-4 concentrations in the subμM range, while higher concentrations fully inhibited MPP^+ transport (IC_{50} 7.1 μM).

3.4. Interactions of isocyanines and adrenergic ligands with multiple subtypes of cloned, human and native, rat α_1 and α_2-adrenoceptors

D24 displayed affinity for all subtypes of hα_1-adrenoceptors, but with significantly lower affinity for the hα_{1B}-adrenoceptor over other hα_1-adrenoceptors. It also displayed comparable affinity for native rat α_1-adrenoceptors (Table 1). The affinities of D24 for hα_2-adrenoceptor subtypes and for native rat α_{2D}-adrenoceptors were overall higher than for hα_1-adrenoceptors, with a mild but significant preference for hα_{2A}-adrenoceptors. AAC291 displayed a very similar profile to D24 with significantly lower affinity for hα_{1B}- *vs* other hα_{1A} - and hα_{1D}-adrenoceptors. Also like D24, it showed a modest but significant preference for hα_{2A}-adrenoceptor over other hα_2-adrenoceptor subtypes. Overall, its affinities at hα_1-adrenoceptors were somewhat higher than for D24, and its affinities at hα_2-adrenoceptor subtypes, somewhat lower, though differences were not significant statistically (Table 1). Overall, AAC301 revealed a profile across hα_1- and hα_2-adrenoceptor subtypes similar to those of D24 and AAC291 (Table 1).

As previously reported (Assari et al., 2003; Giardina et al., 1996), AH11110A displayed high affinity for all hα_1-adrenoceptor subtypes, with significantly higher affinity at hα_{1B}-adrenoceptors, followed by hα_{1A}- then hα_{1D}-adrenoceptors (Table 1). In comparison, its affinities at hα_2-adrenoceptor subtypes were clearly lower. RWJ52353 revealed lower affinities at hα_1-adrenoceptors than AH11110A but, conversely, higher affinities at hα_2-adrenoceptors (Table 1). It showed a modest preference for hα_{1A}-adrenoceptors over other hα_1-adrenoceptor subtypes (Table 1). DSP-4 was inactive at hα_1-adrenoceptors but, surprisingly, interacted at hα_2-adrenoceptors with a significant preference for the 2B subtype.

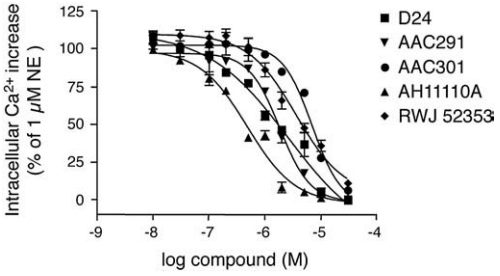

Fig. 4. Antagonist properties at hα_{1A}-adrenoceptors. The increase of intracellular Ca^{2+} is expressed as a percentage of the maximal increase acquired with norepinephrine (1 μM NE, defined as 100%). Data are expressed as means of duplicate determinations from representative experiments repeated on at least two independent occasions. For pK_b values, see Table 2.

Antagonist properties at hα_{1A}-adrenoceptors were evaluated using measurements of intracellular Ca^{2+} concentrations by fluorometry. D24, AAC291, AAC301 and AH11110A all behaved as pure antagonists at hα_1-adrenoceptors and induced between 99 and 107% inhibition of the increase in Ca^{2+} concentrations obtained in the presence of 1 μM norepinephrine (Fig. 4). These compounds exhibited however contrasting potencies, and AAC301 was the least potent agent (significantly $P<0.05$ less potent than the other agents) (Table 2). By contrast to the above-mentioned drugs, RWJ52353 was a partial agonist at hα_{1A}-adrenoceptors (25% efficacy). In a procedure of [^{35}S]GTPγS binding, D24, AAC291, AAC301, AH11110A and RWJ52353 all showed antagonist properties at each subtype of hα_2-adrenoceptor (Table 2). Overall, D24 and AAC291 were the most potent ligands and AH11110A the least potent ($P<0.05$ *vs* D24 and AAC291 for all subtypes). As in binding studies, AH11110A was significantly more potent as an antagonist of hα_{1A}-adrenoceptors than in blocking hα_2-adrenoceptors.

3.5. Interactions of isocyanines and adrenergic ligands with cloned human monoamine transporters

D24 displayed low affinity for both human dopamine transporter (hDAT) and hNET, but its affinity for human serotonin transporter (hSERT) was significantly higher (Table 1). By analogy, the affinities of AAC291 and AAC 301 for hDAT and hNET were low and, in contrast to D24, they also displayed only *low* affinities for hSERT. The affinities of both AH11110A and RWJ52353 for each class of monoamine

Table 1
Affinities (pK_i values) of compounds at human monoamine transporters and at multiple subtypes of hα_1, hα_2, rα_1 and rα_{2D} adrenoceptors.

Receptor	Cells	Radioligand (nM)	D24	AAC291	AAC301	AH11110A	RWJ52353	DSP-4
hα_{1A}	CHO	[^{3}H] Prazosin (0.3)	6.90 ± 0.02	7.09 ± 0.01	6.86 ± 0.18	7.57 ± 0.19	6.64 ± 0.11*	nd
hα_{1B}	CHO	[^{3}H] Prazosin (0.3)	6.18 ± 0.00*	6.40 ± 0.01*	6.85 ± 0.21	8.02 ± 0.21*	6.07 ± 0.09	nd
hα_{1D}	CHO	[^{3}H] Prazosin (0.3)	6.65 ± 0.04	6.89 ± 0.05	7.07 ± 0.18	6.81 ± 0.05	6.04 ± 0.14	nd
hα_{2A}	CHO	[^{3}H] RX 821002 (1)	7.55 ± 0.03*	7.38 ± 0.04*	7.12 ± 0.04	6.12 ± 0.01	6.49 ± 0.05	5.65 ± 0.14
hα_{2B}	CHO	[^{3}H] RX 821002 (2)	6.86 ± 0.08	6.74 ± 0.20	6.44 ± 0.03	5.30 ± 0.05	5.85 ± 0.05	7.11 ± 0.04*
hα_{2C}	CHO	[^{3}H] RX 821002 (1)	7.18 ± 0.01	7.08 ± 0.02	7.04 ± 0.05	6.01 ± 0.06	6.19 ± 0.08	5.79 ± 0.07
rα_1	Frontal cortex	[^{3}H] Prazosin (0.5)	6.64 ± 0.00	6.92 ± 0.02	6.82 ± 0.01	7.23 ± 0.20	5.58 ± 0.04	6.06 ± 0.13
rα_{2D}	Frontal cortex	[^{3}H] RX 821002 (1)	7.19 ± 0.02	7.56 ± 0.01	6.73 ± 0.01	6.27 ± 0.06	6.46 ± 0.01	<5
hSERT	HEK293	[^{3}H] Citalopram (2)	6.61 ± 0.06*	<5	5.44 ± 0.02	5.59 ± 0.05	<5	<5
hNET	MDCK	[^{3}H] Nisoxetine (2)	5.38 ± 0.02	<5	5.69 ± 0.10	5.86 ± 0.01	5.67 ± 0.11	<5
hDAT	CHO	[^{3}H] GBR12935 (1)	5.60 ± 0.33	<5	5.04 ± 0.01	<5	<5	<5

Data are means ± S.E.M. of two to three determinations, each one performed in duplicate. SERT, serotonin transporter; NET, norepinephrine transporter; DAT, dopamine transporter; h, human; r, rat; D24: 1,1′-diisopropyl-2,4-cyanine iodide (disprocynium24). See text for further details.
* $P<0.05$ indicates significance of differences *vs* other hα_1 or hα_2 subtypes, and for serotonin *vs* norepinephrine and dopamine transporters.

Table 2
Antagonist properties at multiple subtypes of $h\alpha_2$ receptors.

	$h\alpha_{2A}$	$h\alpha_{2B}$	$h\alpha_{2C}$	$h\alpha_A$
D24	7.15 ± 0.20	7.07 ± 0.14	7.77 ± 0.07	7.12 ± 0.02
AAC 291	7.47 ± 0.22	6.93 ± 0.07	6.99 ± 0.13	7.15 ± 0.15
AAC301	7.00 ± 0.04	6.62 ± 0.00	6.86 ± 0.00	6.38 ± 0.08
AH 11110	5.85 ± 0.10	5.38 ± 0.07	5.65 ± 0.13	7.27 ± 0.03*
RWJ 52353	6.20 ± 0.07	5.31 ± 0.16	5.40 ± 0.15	6.57 ± 0.27
DSP-4	5.91 ± 0.11	6.54 ± 0.07	5.78 ± 0.18	nd

Antagonist properties at subtypes of $h\alpha_2$ and at $h\alpha_{1A}$ receptors were determined by [^{35}S]GTPγS binding and by measurement of intracellular Ca^{2+} concentrations, respectively. Values are pK_b. Data are means ± S.E.M. of two determinations, each one performed in duplicate. See text for further details.

* $P<0.05$ indicates significance of differences to affinities for $h\alpha_{2A}$-adrenoceptor subtypes.

transporter were modest. DSP-4 displayed low affinity for hNET, hSERT and hDAT (Table 1).

3.6. Mechanisms of activation of hOCT2-mediated [^{3}H]MPP$^+$ transport by low concentrations of D24

To explore the mechanisms underlying the activation of hOCT2 transport by low concentrations of inhibitors, we chose to focus on the high-affinity prototypical OCT inhibitor, D24, and first tested whether irreversible binding might be implicated. This was done by examining the effect on hOCT2 transport of washing with the transport buffer (3 times during 15 min) after pre-incubation. Following this washing step, the inhibition curve was essentially identical to that obtained without washing (Fig. 5A), indicating that activation could take place through irreversible binding of the ligand, as is the case for inhibition.

Grundemann et al. (2002) previously showed for rOCT3 that the activating effect of D24 (as well as cimetidine) depends upon the nature of the substrate. Therefore, the putative substrate-dependency of hOCT2 activation by D24 was tested using serotonin (5-HT), a preferred substrate of this transporter. Contrarily to [^{3}H]MPP$^+$, when [^{3}H]5-HT was employed, increasing concentrations of D24 monophasically inhibited transport with an IC_{50} below 10 nM, and in the absence of any activation (Fig. 5B). The activating effect of low concentrations of D24 on hOCT2-mediated transport is, thus, dependent upon the substrate internalized.

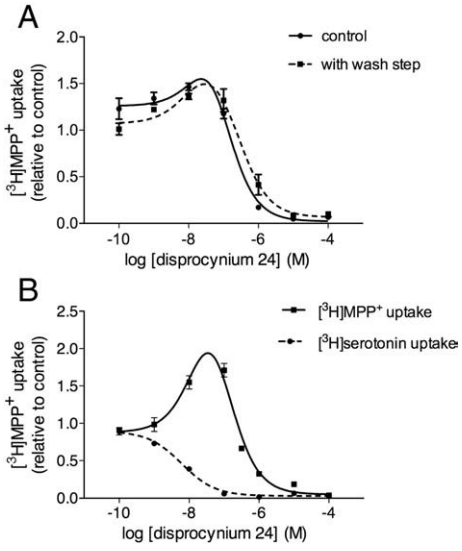

Fig. 5. Influence of "washing" and substrate upon activation of hOCT2-mediated transport by low concentrations of D24. Panel A), [^{3}H]MPP$^+$ (25 nM) uptake was determined for 15 min at 37 °C in the presence of increasing concentrations of D24 with or without washing (15 min) after the pre-incubation period. Panel B), the uptake of [^{3}H]5-HT (100 nM) and [^{3}H]MPP$^+$ (25 nM) was evaluated for 15 min at 37 °C in the presence of increasing concentrations of D24. Data are expressed as the arithmetic means ± S.E.M. of control (in the absence of inhibitors).

Since protein kinase pathways have been shown to modulate OCT activity (see Discussion), we explored whether they may be implicated in the activation of hOCT2-mediated transport at low concentrations of D24. We investigated the effects of inhibition of protein kinase C (PKC), protein kinase A (PKA) and the calmodulin kinase II complex (CaMKII), using the selective inhibitors Ro 32-0432, KT 5720 and KN 93, respectively. These inhibitors were tested in the presence of two concentrations of D24, 10 nM, which produces maximum activation, and 10 μM, which completely inhibits transport. In the absence of D24, Ro 32-0432 did not modify hOCT2-mediated uptake. One μM Ro 32-0432 failed to influence activation of [^{3}H]MPP$^+$ uptake by a low concentration of D24 (10 nM) whereas, at a concentration of 10 μM, Ro-32-0432 blocked this facilitation. Inhibitory concentrations of D24 (10 μM) completely abolished uptake, irrespective of the presence or concentration of Ro 32-0432 (Fig. 6A). KT 5720 did not modify basal hOCT2-mediated [^{3}H]MPP$^+$ uptake, nor uptake in the presence of D24 at activating or inhibitory concentrations (Fig. 6B). Likewise, KN93 did not alter significantly the influence of D24 upon transport (Fig. 6C). These results indicate that D24-mediated activation is principally dependent upon PKC activation. Underpinning these observations, in a separate study of the full dose–response curve for D24, the activation peak was abolished in the presence of 10 μM Ro 32-0432 (Fig. 6D).

4. Discussion

4.1. Actions of D24 and isocyanine derivatives at OCTs and α_1-adrenoceptors

This present study demonstrates that several classes of "adrenergic" ligand functionally interact with all three subtypes of OCT, underpinning and amplifying previous observations of promiscuity amongst pharmacophores interacting with α_1- and α_2-adrenoceptors on the one hand, and OCT transporters on the other. Cyanine dyes themselves behave as α_1-adrenoceptor antagonists, while a number of other α- and β-adrenoceptor agonists and antagonists inhibit specific OCT subtypes (Hayer-Zillgen et al., 2002; Koepsell et al., 2007). It was therefore important to evaluate and compare the relative affinity of these compounds for OCTs and adrenoceptors to better interpret their actions *in vivo*.

Both D24 and the two other isocyanine derivatives produced two different types of effect on OCT-mediated [^{3}H]MPP$^+$ uptake. D24, AAC291 and AAC301 all inhibited hOCT3-mediated transport in a standard concentration-dependent manner, as did D24 for rOCT1- and hOCT1-mediated transport. In contrast, low concentrations of all three agents enhanced OCT2-mediated transport, while higher concentrations were inhibitory, generating a complex bell-shaped profile. Neither of the two D24 analogues tested was more potent for inhibiting OCT transport than D24 itself, but they showed more robust activating properties, stimulating transport by up to threefold. Such an activating effect of low concentrations of D24 was initially reported for rOCT3 and mimicked by MPP$^+$ as well as other OCT substrates like cimetidine (Grundemann et al., 2002). Here, we show that this phenomenon extends to OCT1 and OCT2, of both rat and human species.

D24 has been shown to interact with native α_1-adrenoceptors in rat myocardium (240 nM) (Russ et al., 1996) and the present study provides the first comprehensive evaluation of its interaction with all known subtypes of $h\alpha_1$-adrenoceptors and $h\alpha_2$-adrenoceptors at which it displayed modest affinity and behaved as an antagonist (Table 1). Obviously, these effects should be carefully considered in future studies exploiting D24 as a tool for characterisation of the influence of OCTs upon monoaminergic and other pathways

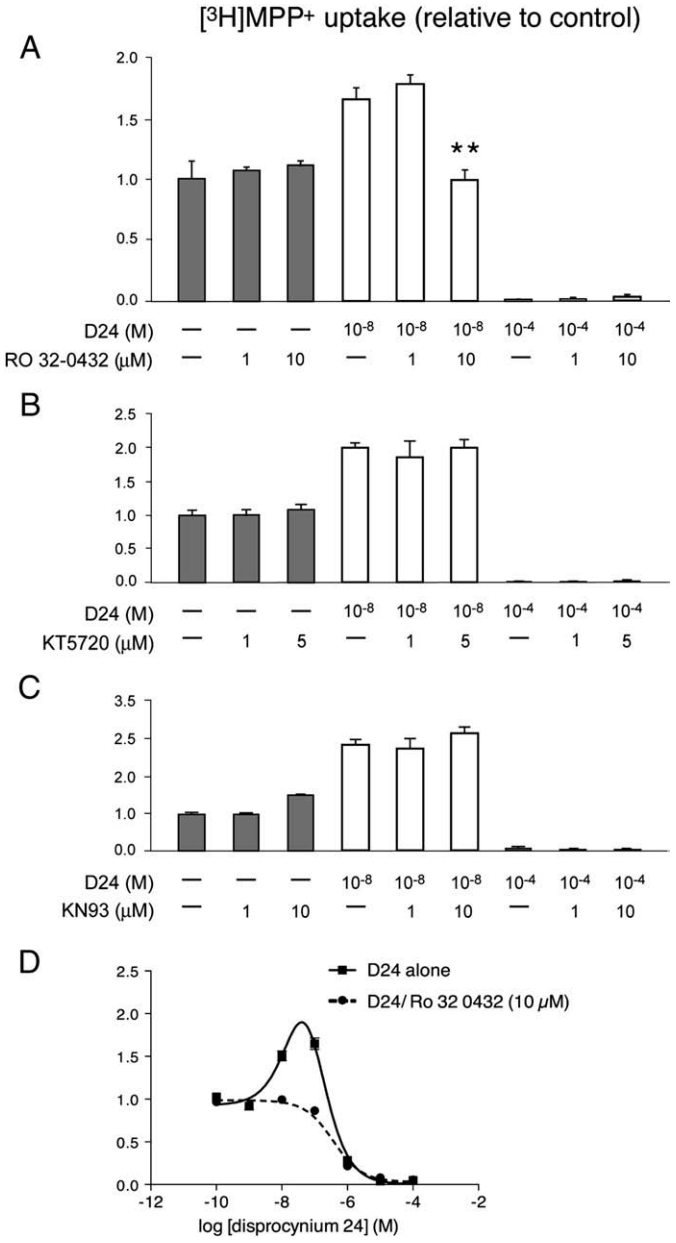

Fig. 6. Influence of inhibitors of protein kinase C (PKC), protein kinase A (PKA) and calmodulin kinase II (CaMKII) upon activation of hOCT2-mediated transport by low concentrations of D24. Selective PKC, PKA, and Ca/CaMKII protein inhibitors were tested for their effect on hOCT2-mediated [^{3}H]MPP$^+$ transport in stably transfected HEK293 cells. For panels A to C, [^{3}H]MPP$^+$ (25 nM) uptake was evaluated for 15 min at 37 °C in the presence of two concentrations of D24 (one low, facilitatory and one high, inhibitory), plus the specified inhibitor which was added during the pre-incubation period. In panel d, a full range of concentrations of D24 was evaluated. Panel a: 1 and 10 μM of the specific PKC inhibitor, Ro 32-0432. Panel B), 1 and 5 μM of the specific PKA inhibitor, KT 5720. Panel C), 1 and 10 μM of the specific Ca/CaMKII protein inhibitor, KN 96. Panel D): 10 μM of Ro 32-043. Data are expressed as the arithmetic means ± S.E.M.s of control uptake (in the absence of inhibitors). **Indicates the significance ($P<0.01$, Student's two-tailed t-test) of the differences between inhibitor and control values for D24 at a concentration of 10^{-8}M.

(Invernizzi and Garattini, 2004; Millan et al., 2000). In addition, the present study also reveals modest affinity of D24 for SERT, which supports interest in this compound as a basis for developing "dual-active" ligands for the treatment of depression and other disorders (Millan, 2006). AAC291 and AAC301, with lower affinity for SERT, clearly present an advantage as tools for investigation of the influence of OCTs upon serotonergic neurotransmission.

4.2. Actions of the adrenergic ligands AH11110A and RWJ52353 at OCTs and α-adrenoceptors

As also shown herein, the preferential α_{1B}-adrenoceptor antagonist, AH11110A concentration-dependently inhibited [^{3}H]MPP$^+$ transport by all 3 OCT subtypes, acting most potently at OCT1. Further, an activating component was seen for rOCT1 and rOCT2 as well as hOCT2. In contrast to other agents tested herein, the α_2-adrenoceptor agonist, RWJ52353, only weakly attenuated transport at OCTs, though it elicited a striking activating effect (threefold) at rOCT3 and also enhanced transport at hOCT2. Collectively, these data indicate that these structurally distinct α-adrenoceptor agents interact with OCTs, possibly reflecting their protonation at physiological pH.

The present data corroborate the modest preference of AH11110A for α_{1B}- over other adrenoceptor subtypes and demonstrate antagonist properties using a *common* measure of Ca^{2+} flux: these findings extend older studies where its actions at a range of α_1-adrenoceptor receptor subtypes from various species were examined using diverse functional measures (Assari et al., 2003; Eltze et al., 2001; Giardina et al., 1996). As regards RWJ52353, its affinities for hα_{2A}- and hα_{2B}-adrenoceptors correspond closely to those documented by Ross et al. (2000) (who did not examine hα_{2C}-adrenoceptors), whereas its affinity for rα_{2D}-adrenoceptors herein was far lower than originally reported. This difference can be explained by the common use of the radiolabelled *antagonist*, [^{3}H]RX821,002 to label human sites, whereas, in contrast to [^{3}H]RX821,002/rat cortex in this study, Ross et al. (2000) determined affinities at rα_{2D}-adrenoceptors using a radiolabelled *agonist*, [^{3}H]para-aminoclonidine, and the spinal cord. Inasmuch as RWJ52353 appears on the basis of *in vivo* observations to be an *agonist* at rα_{2D}-adrenoceptors, its affinity at these sites was strongly favoured. Hence, employing comparable binding procedures, RWJ52353 is not a preferential ligand of rα_{2D}-adrenoceptors. Moreover, to our surprise, in a GTPysS binding assay, it behaved as an *antagonist* at hα_2-adrenoceptors in contrast to its agonist actions in rats (Ross et al., 2000). It is possible that a more sensitive downstream measure of efficacy (like ERK1/2 phosphorylation) might have picked up agonist properties. Nonetheless, these observations may explain why this drug is no longer in development as an analgesic.

4.3. Molecular mechanisms involved in OCT2-mediated transport facilitation by low concentrations of D24

Activation of hOCT2-mediated MPP$^+$ transport by low concentrations of D24 is substrate-dependent and blocked by the PKC inhibitor Ro 32-0432, transforming its effects into a monophasic inhibition curve. Our findings also suggest that D24 irreversibly binds to hOCT2, since its activating and inhibiting effect on MPP$^+$ transport remained unchanged after washing. Several other lines of data support the hypothesis that activation is mediated by an interaction of D24 with OCTs and strongly argue against a role at α-adrenoceptors. *First*, all ligands tested interacted with α_1- and α_2-adrenoceptors but did not act in the same manner at various OCT subtypes. *Second*, activation was found previously with OCT substrates *not* interacting with adrenoceptors, such as cimetidine and DMPBI (Grundemann et al., 2002). *Third*, the transfected cell line HEK293 used in the experiments does not express α_1 or α_2-adrenoceptors (Lei et al., 2002; Olli-Lahdesmaki et al., 2004).

The mechanisms of interaction of OCTs with substrates and inhibitors have not been fully elucidated. A series of studies collectively argue for the existence of partially distinct and overlapping sites for ligand binding and translocation (Ciarimboli et al., 2005) and in particular, a model featuring one or more high-affinity sites and a low-affinity site, the latter required for transition from an outward to an inward-oriented conformation and substrate translocation (Gorbunov et al., 2008). Such multiple co-existing binding sites allowing allosteric structural changes could explain the activating effect of D24 and other

compounds (Grundemann et al., 2002). Binding of low concentrations of D24 at a high-affinity site would induce an allosteric transition favouring substrate interaction and/or transport at the low-affinity site, while higher D24 concentrations would directly block the low-affinity site and substrate translocation. This model is also compatible with the substrate-dependence of the activating effect of D24 and argues in favour of distinct and partially overlapping binding sites also for MPP^+ and serotonin. A similar dependence of activation on the nature of substrate was found previously (Grundemann et al., 2002), in that low concentrations of D24 activated the transport of MPP^+ but not cimetidine and DMPBI by rOCT3.

Previous *ex vivo* experiments with the model substrate 4-(4-dimethylaminostyryl)-N-methylpyridinium (ASP^+) have also shown that OCTs activity can be regulated, depending on subtype, by multiple phosphorylation pathways, including PKC, PKA and Ca/CaMKII. In the case of rOCT1, PKC phosphorylation sites located in the binding pocket were shown to differentially contribute to the affinity for diverse substrates (Ciarimboli et al., 2005 and references within). Here, we show that the activation of hOCT2-mediated MPP^+ transport by low concentrations of D24 depends on PKC activation. Distinct phosphorylation patterns could thus account for the fact that only some OCT subtypes are sensitive to the activating effect of D24. Possibly D24 and, by extension other activating compounds, induce a conformational change leading to the unmasking of PKC recognition motifs on hOCT2 (Ciarimboli et al., 2005; Ciarimboli and Schlatter, 2005): phosphorylation would then lead to a modification in activity. Alternatively, phosphorylation of specific motifs by PKC could induce conformational changes in the transporter favouring interaction with low concentration of D24 and increased transporter activity. Further experiments are needed to elucidate the precise relationship between the phosphorylation state of the transporter and the activation process by low concentrations of inhibitors.

4.4. Action of the adrenergic neurotoxin DSP-4 at OCTs, monoamine transporters and α-adrenoceptors

Interestingly the present studies revealed an unexpected and particularly strong influence of another category of compound, the neurotoxin DSP-4, upon MPP^+ transport by all 3 classes of OCT. Mechanisms underlying the effects of DSP-4 on highly-vulnerable locus coeruleus adrenergic neurons are, perhaps surprisingly, not fully understood (Fritschy and Grzanna, 1991; Lyles and Callingham, 1981). In this study, DSP-4 actions at OCTs were observed over a range of concentrations comparable to those (200–400 nM) reported active for native rat NETs, as estimated by norepinephrine reuptake into synaptosomes (Zaczek et al., 1990), but intriguingly no interaction with human NET could be detected herein in binding studies, even at 10 fold higher concentrations. Whether this reflects a species difference, contrasting methods for determining potencies, use of a different radioligand, multiple binding sites on SERTs, the fact that synaptosomes are not a pure preparation of NETs, or other factors, remains to be established (Millan, 2006). In any event OCTs, and in particular OCT2, could be additional targets for DSP-4 in the brain, an interaction which could underlie some of its neurochemical or neurotoxic effects (Lyons et al., 1989). In this regard, the present study also revealed unanticipated actions of DSP-4 at α_1- and $h\alpha_2$-adrenoceptors (though not at $r\alpha_{2D}$-adrenoceptors), which should also be taken into account in its utilisation as a neurotoxic tool or *in vitro* studies.

4.5. Potential functional and therapeutic significance

Taken together, the present studies have some interesting physiological and therapeutic implications as regards the functional roles of OCTs and adrenergic neurotransmission, and the actions and availability of drugs.

The enhancement of OCT activity by low concentrations of inhibitors or substrates appears to be a common yet OCT subtype- and species-dependent phenomenon. To date, mainly high-affinity exogenous compounds such as D24, the isocyanine analogues studied herein, adrenergic ligands, DSP-4, cimetidine, MPP^+ and DMBPI have been shown to provoke this effect (Grundemann et al., 2002). However, other as yet unidentified drugs may prove to act similarly and, based on the present observations, it would be interesting to pursue this line of research in the hope of finding more potent agents that discriminate OCT subtypes and display improved selectivity *vs* $\alpha_{1/2}$-adrenoceptors and monoamine transporters.

Indeed, the present work identifies several pharmacophores that recognise both OCTs *and* α-adrenoceptors, two families that share a close anatomical and functional relationship in sympathetically-innervated tissues and the brain. It raises the intriguing possibility that some adrenergic ligands may act concomitantly on OCT-mediated catecholamine uptake and at $\alpha_{1/2}$-adrenoceptors, either in a cooperative or opposite manner, *in vivo* in sympathetically-innervated tissues or the CNS. At low doses, antihypertensive drugs acting as α-adrenoceptor antagonists may, for instance, reinforce their actions by increasing norepinephrine clearance through OCTs (Hayer-Zillgen et al., 2002). In this light, it would be instructive to assess whether other adrenergic drugs, in particular therapeutically-employed agents, are capable of activating OCT transport at low concentrations. In addition, OCTs could potentially regulate the pharmacokinetics and action of these ligands if these are also transported by OCTs, a possibility which remains to be investigated.

The activating and inhibitory properties of compounds characterised herein may also have implications for human disease as well as positive and negative drug interactions. Due to their strategic localization in peripheral organs, OCTs tightly control the pharmacokinetics of a variety of compounds, such as antimalarials, antidiabetics, antiviral agents, cytostatics and H_2 receptor antagonists (Koepsell et al., 2007). Alterations of OCT activity may enhance the sensitivity to endogenous and environmental neurotoxins and contribute to neurodegenerative disease. In the brain, OCT3 was recently shown to play a pivotal role in neurodegeneration in dopaminergic pathways (Cui et al., 2009). Our study suggests that these diverse functions could be affected by the administration of low concentrations of adrenergic compounds that act at OCTs. In particular, genetic variants of hOCT2 have been associated with reduced renal or tubular clearance of the biguanide metformin, a drug used to treat type-II diabetes (Chen et al., 2009; Song et al., 2008; Wang et al., 2008; Zolk et al., 2009). Shu et al. (2003) have raised the possibility that an imbalance in OCT1 *vs* OCT2 activity could affect plasma metformin levels, lead to its accumulation in the liver and result in lactic acidosis, a side-effect which could be prevented by increasing kidney excretion through OCT2. The isocyanine derivatives tested in the present study selectively enhance hOCT2 activity and have the potential in the subμM range to activate hOCT2 with a limited effect on hOCT1 and hOCT3. These or other compounds with similar but improved profiles represent an interesting therapeutic approach to selectively stimulate OCT2-driven clearance in the kidney without interfering with the activity of the other transporters.

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References

Amphoux, A., Vialou, V., Drescher, E., Bruss, M., Mannoury La Cour, C., Rochat, C., Millan, M.J., Giros, B., Bonisch, H., Gautron, S., 2006. Differential pharmacological *in vitro* properties of organic cation transporters and regional distribution in rat brain. Neuropharmacology 50, 941–952.

Asaka, J., Terada, T., Okuda, M., Katsura, T., Inui, K., 2006. Androgen receptor is responsible for rat organic cation transporter 2 gene regulation but not for rOCT1 and rOCT3. Pharm. Res. 23, 697–704.

Assari, T., Cox, S., Munday, M.R., Pearce, B., 2003. Regulation of alpha(1)-adrenoceptor-linked phosphoinositide metabolism in cultured glia: involvement of protein phosphatases and kinases. Cell. Signal. 15, 403–412.

Baganz, N.L., Horton, R.E., Calderon, A.S., Owens, W.A., Munn, J.L., Watts, L.T., Koldzic-Zivanovic, N., Jeske, N.A., Koek, W., Toney, G.M., Daws, L.C., 2008. Organic cation transporter 3: keeping the brake on extracellular serotonin in serotonin-transporter-deficient mice. Proc. Natl. Acad. Sci. U. S. A. 105, 18976–18981.

Chen, Y., Li, S., Brown, C., Cheatham, S., Castro, R.A., Leabman, M.K., Urban, T.J., Chen, L., Yee, S.W., Choi, J.H., Huang, Y., Brett, C.M., Burchard, E.G., Giacomini, K.M., 2009. Effect of genetic variation in the organic cation transporter 2 on the renal elimination of metformin. Pharmacogenet. Genomics 19, 497–504.

Cheng, Y., Prusoff, W.H., 1973. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099–3108.

Ciarimboli, G., Schlatter, E., 2005. Regulation of organic cation transport. Pflugers Arch. 449, 423–441.

Ciarimboli, G., Koepsell, H., Iordanova, M., Gorboulev, V., Durner, B., Lang, D., Edemir, B., Schroter, R., Van Le, T., Schlatter, E., 2005. Individual PKC-phosphorylation sites in organic cation transporter 1 determine substrate selectivity and transport regulation. J. Am. Soc. Nephrol. 16, 1562–1570.

Cui, M., Aras, R., Christian, W.V., Rappold, P.M., Hatwar, M., Panza, J., Jackson-Lewis, V., Javitch, J.A., Ballatori, N., Przedborski, S., Tieu, K., 2009. The organic cation transporter-3 is a pivotal modulator of neurodegeneration in the nigrostriatal dopaminergic pathway. Proc. Natl. Acad. Sci. U. S. A. 106, 8043–8048.

Daws, L.C., 2009. Unfaithful neurotransmitter transporters: focus on serotonin uptake and implications for antidepressant efficacy. Pharmacol. Ther. 121, 89–99.

Eisenhofer, G., McCarty, R., Pacak, K., Russ, H., Schomig, E., 1996. Disprocynium24, a novel inhibitor of the extraneuronal monoamine transporter, has potent effects on the inactivation of circulating noradrenaline and adrenaline in conscious rat. Naunyn Schmiedebergs Arch. Pharmacol. 354, 287–294.

Eltze, M., Konig, H., Ullrich, B., Grebe, T., 2001. Failure of AH11110A to functionally discriminate between alpha(1)-adrenoceptor subtypes A, B and D or between alpha(1)- and alpha(2)-adrenoceptors. Eur. J. Pharmacol. 415, 265–276.

Fritschy, J.M., Grzanna, R., 1991. Selective effects of DSP-4 on locus coeruleus axons: are there pharmacologically different types of noradrenergic axons in the central nervous system? Prog. Brain Res. 88, 257–268.

Giardina, D., Crucianelli, M., Romanelli, R., Leonardi, A., Poggesi, E., Melchiorre, C., 1996. Synthesis and biological profile of the enantiomers of [4-(4-amino-6, 7-dimethoxyquinazolin-2-yl)-cis-octahydroquinoxalin- 1-yl]furan-2-ylmethanone (cyclazosin), a potent competitive alpha 1B- adrenoceptor antagonist. J. Med. Chem. 39, 4602–4607.

Gorbunov, D., Gorboulev, V., Shatskaya, N., Mueller, T., Bamberg, E., Friedrich, T., Koepsell, H., 2008. High-affinity cation binding to organic cation transporter 1 induces movement of helix 11 and blocks transport after mutations in a modeled interaction domain between two helices. Mol. Pharmacol. 73, 50–61.

Graefe, K.H., Friedgen, B., Wolfel, R., Bossle, F., Russ, H., Schomig, E., 1997. 1, 1′-Diisopropyl-2, 4′-cyanine (disprocynium24), a potent uptake2 blocker, inhibits the renal excretion of catecholamines. Naunyn Schmiedebergs Arch. Pharmacol. 356, 115–125.

Grundemann, D., Babin-Ebell, J., Martel, F., Ording, N., Schmidt, A., Schomig, E., 1997. Primary structure and functional expression of the apical organic cation transporter from kidney epithelial LLC-PK1 cells. J. Biol. Chem. 272, 10408–10413.

Grundemann, D., Schechinger, B., Rappold, G.A., Schomig, E., 1998. Molecular identification of the corticosterone-sensitive extraneuronal catecholamine transporter. Nat. Neurosci. 1, 349–351.

Grundemann, D., Koschker, A.C., Haag, C., Honold, C., Zimmermann, T., Schomig, E., 2002. Activation of the extraneuronal monoamine transporter (EMT) from rat expressed in 293 cells. Br. J. Pharmacol. 137, 910–918.

Grzanna, R., Berger, U., Fritschy, J.M., Geffard, M., 1989. Acute action of DSP-4 on central norepinephrine axons: biochemical and immunohistochemical evidence for differential effects. J. Histochem. Cytochem. 37, 1435–1442.

Hayer-Zillgen, M., Bruss, M., Bonisch, H., 2002. Expression and pharmacological profile of the human organic cation transporters hOCT1, hOCT2 and hOCT3. Br. J. Pharmacol. 136, 829–836.

Hidaka, H., Inagaki, M., Kawamoto, S., Sasaki, Y., 1984. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry 23, 5036–5041.

Invernizzi, R.W., Garattini, S., 2004. Role of presynaptic alpha2-adrenoceptors in antidepressant action: recent findings from microdialysis studies. Prog. Neuropsychopharmacol. Biol. Psychiatry 28, 819–827.

Iversen, L.L., 1965. The uptake of catecholamines at high perfusion concentrations in the rat isolated heart: a novel catechol amine uptake process. Br. J. Pharmacol. 25, 18–33.

Jonker, J.W., Wagenaar, E., Mol, C.A., Buitelaar, M., Koepsell, H., Smit, J.W., Schinkel, A.H., 2001. Reduced hepatic uptake and intestinal excretion of organic cations in mice with a targeted disruption of the organic cation transporter 1 (Oct1 [Slc22a1]) gene. Mol. Cell. Biol. 21, 5471–5477.

Jonker, J.W., Wagenaar, E., Van Eijl, S., Schinkel, A.H., 2003. Deficiency in the organic cation transporters 1 and 2 (Oct1/Oct2 [Slc22a1/Slc22a2]) in mice abolishes renal secretion of organic cations. Mol. Cell. Biol. 23, 7902–7908.

Koepsell, H., Lips, K., Volk, C., 2007. Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. Pharm. Res. 24, 1227–1251.

Lei, B., Zhang, Y., Han, C., 2002. Changes in mRNA expression induced by sustained noradrenaline stimulation are different for alpha1-adrenoceptor subtypes in HEK293 cells. Clin. Exp. Pharmacol. Physiol. 29, 1084–1090.

Lyles, G.A., Callingham, B.A., 1981. The effect of DSP-4 (N-[2-chloroethyl]-N-ethyl-2-bromobenzylamine) on monoamine oxidase activities in tissues of the rat. J. Pharm. Pharmacol. 33, 632–638.

Lyons, W.E., Fritschy, J.M., Grzanna, R., 1989. The noradrenergic neurotoxin DSP-4 eliminates the coeruleospinal projection but spares projections of the A5 and A7 groups to the ventral horn of the rat spinal cord. J. Neurosci. 9, 1481–1489.

Mamiya, N., Goldenring, J.R., Tsunoda, Y., Modlin, I.M., Yasui, K., Usuda, N., Ishikawa, T., Natsume, A., Hidaka, H., 1993. Inhibition of acid secretion in gastric parietal cells by the Ca2+/calmodulin-dependent protein kinase II inhibitor KN-93. Biochem. Biophys. Res. Commun. 195, 608–615.

Millan, M.J., 2006. Multi-target strategies for the improved treatment of depressive states: conceptual foundations and neuronal substrates, drug discovery and therapeutic application. Pharmacol. Ther. 110, 135–370.

Millan, M.J., Lejeune, F., Gobert, A., 2000. Reciprocal autoreceptor and heteroreceptor control of serotonergic, dopaminergic and noradrenergic transmission in the frontal cortex: relevance to the actions of antidepressant agents. J. Psychopharmacol. 14, 114–138.

Millan, M.J., Cussac, D., Gobert, A., Lejeune, F., Rivet, J.M., Mannoury La Cour, C., Newman-Tancredi, A., Peglion, J.L., 2004. S32504, a novel naphtoxazine agonist at dopamine D3/D2 receptors: I. Cellular, electrophysiological, and neurochemical profile in comparison with ropinirole. J. Pharmacol. Exp. Ther. 309, 903–920.

Minuesa, G., Volk, C., Molina-Arcas, M., Gorboulev, V., Erkizia, I., Arndt, P., Clotet, B., Pastor-Anglada, M., Koepsell, H., Martinez-Picado, J., 2009. Transport of lamivudine [(−)-beta-L-2′, 3′-dideoxy-3′-thiacytidine] and high-affinity interaction of nucleoside reverse transcriptase inhibitors with human organic cation transporters 1, 2, and 3. J. Pharmacol. Exp. Ther. 329, 252–261.

Nakayama, H., Kitaichi, K., Ito, Y., Hashimoto, K., Takagi, K., Yokoi, T., Takagi, K., Ozaki, N., Yamamoto, T., Hasegawa, T., 2007. The role of organic cation transporter-3 in methamphetamine disposition and its behavioral response in rats. Brain Res. 1184, 260–269.

Newman-Tancredi, A., Nicolas, J.P., Audinot, V., Gavaudan, S., Verriele, L., Touzard, M., Chaput, C., Richard, N., Millan, M.J., 1998. Actions of alpha2 adrenoceptor ligands at alpha2A and 5-HT1A receptors: the antagonist, atipamezole, and the agonist, dexmedetomidine, are highly selective for alpha2A adrenoceptors. Naunyn Schmiedebergs Arch. Pharmacol. 358, 197–206.

Olli-Lahdesmaki, T., Tiger, M., Vainio, M., Scheinin, M., Kallio, J., 2004. Ligand-induced alpha2-adrenoceptor endocytosis: relationship to Gi protein activation. Biochem. Biophys. Res. Commun. 321, 226–233.

Ross, T.M., Jetter, M.C., McDonnell, M.E., Boyd, R.E., Connelly, C.D., Martinez, R.P., Lewis, M.A., Codd, E.E., Raffa, R.B., Reitz, A.B., 2000. alpha(2) Adrenoceptor agonists as potential analgesic agents. 2. Discovery of 4-(4-Imidazo)-1, 3-dimethyl-6, 7-dihydrothianaphthene as a high-affinity ligand for the alpha(2D) adrenergic receptor. J. Med. Chem. 43, 765–768.

Russ, H., Engel, W., Schomig, E., 1993. Isocyanines and pseudoisocyanines as a novel class of potent noradrenaline transport inhibitors: synthesis, detection, and biological activity. J. Med. Chem. 36, 4208–4213.

Russ, H., Friedgen, B., Konigs, B., Schumacher, C., Graefe, K.H., Schomig, E., 1996. Pharmacokinetic and alpha 1-adrenoceptor antagonistic properties of two cyanine-type inhibitors of extraneuronal monoamine transport. Naunyn Schmiedebergs Arch. Pharmacol. 354, 268–274.

Shu, Y., Leabman, M.K., Feng, B., Mangravite, L.M., Huang, C.C., Stryke, D., Kawamoto, M., Johns, S.J., DeYoung, J., Carlson, E., Ferrin, T.E., Herskowitz, I., Giacomini, K.M., 2003. Evolutionary conservation predicts function of variants of the human organic cation transporter, OCT1. Proc. Natl. Acad. Sci. U. S. A. 100, 5902–5907.

Song, I.S., Shin, H.J., Shin, J.G., 2008. Genetic variants of organic cation transporter 2 (OCT2) significantly reduce metformin uptake in oocytes. Xenobiotica 38, 1252–1262.

Trendelenburg, U., 1988. The extraneuronal uptake and metabolism of catecholamines. In: Trendelenburg, U., Weiner, N. (Eds.), Handbook of Experimental Pharmacology, Vol. 90/I. Springer, Berlin, pp. 279–319.

Vialou, V., Amphoux, A., Zwart, R., Giros, B., Gautron, S., 2004. Organic cation transporter 3 (Slc22a3) is implicated in salt-intake regulation. J. Neurosci. 24, 2846–2851.

Vialou, V., Balasse, L., Callebert, J., Launay, J.M., Giros, B., Gautron, S., 2008. Altered aminergic neurotransmission in the brain of organic cation transporter 3-deficient mice. J. Neurochem. 106, 1471–1482.

Wang, Z.J., Yin, O.Q., Tomlinson, B., Chow, M.S., 2008. OCT2 polymorphisms and in-vivo renal functional consequence: studies with metformin and cimetidine. Pharmacogenet. Genomics 18, 637–645.

Wilkinson, S.E., Parker, P.J., Nixon, J.S., 1993. Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. Biochem. J. 294 (Pt 2), 335–337.

Zaczek, R., Fritschy, J.M., Culp, S., De Souza, E.B., Grzanna, R., 1990. Differential effects of DSP-4 on noradrenaline axons in cerebral cortex and hypothalamus may reflect heterogeneity of noradrenaline uptake sites. Brain Res. 522, 308–314.

Zhang, L., Dresser, M.J., Gray, A.T., Yost, S.C., Terashita, S., Giacomini, K.M., 1997. Cloning and functional expression of a human liver organic cation transporter. Mol. Pharmacol. 51, 913–921.

Zolk, O., Solbach, T.F., Konig, J., Fromm, M.F., 2009. Functional characterization of the human organic cation transporter 2 variant p. 270Ala>Ser. Drug Metab. Dispos. 37, 1312–1318.

Zwart, R., Verhaagh, S., Buitelaar, M., Popp-Snijders, C., Barlow, D.P., 2001. Impaired activity of the extraneuronal monoamine transporter system known as uptake-2 in Orct3/Slc22a3-deficient mice. Mol. Cell. Biol. 21, 4188–4196.