

Washington University in St. Louis

## Washington University Open Scholarship

---

Undergraduate Theses—Unrestricted

---

Spring 5-15-2015

### Pharmacology of Organic Cation Transporters: Focus on Structure-Function Relationships in OCT3 (SLC22A3)

Dan C. Li

*Washington University in St Louis*

Follow this and additional works at: [https://openscholarship.wustl.edu/undergrad\\_open](https://openscholarship.wustl.edu/undergrad_open)



Part of the [Biochemistry Commons](#), [Biophysics Commons](#), and the [Structural Biology Commons](#)

---

#### Recommended Citation

Li, Dan C., "Pharmacology of Organic Cation Transporters: Focus on Structure-Function Relationships in OCT3 (SLC22A3)" (2015). *Undergraduate Theses—Unrestricted*. 29.

[https://openscholarship.wustl.edu/undergrad\\_open/29](https://openscholarship.wustl.edu/undergrad_open/29)

This Dissertation/Thesis is brought to you for free and open access by Washington University Open Scholarship. It has been accepted for inclusion in Undergraduate Theses—Unrestricted by an authorized administrator of Washington University Open Scholarship. For more information, please contact [digital@wumail.wustl.edu](mailto:digital@wumail.wustl.edu).

WASHINGTON UNIVERSITY IN ST. LOUIS

The College of Arts and Sciences

PHARMACOLOGY OF ORGANIC CATION TRANSPORTERS (OCTs):  
FOCUS ON STRUCTURE-FUNCTION RELATIONSHIPS IN OCT3 (*SLC22A3*)

Research Emphasis Thesis

By

Dan C. Li

Presented to:

The Department of Biology

In partial fulfillment of the requirements for

Honors for the degree of

Bachelor of Arts in Biology

Washington University in St. Louis

Saint Louis, Missouri

March 24, 2015

## ACKNOWLEDGEMENTS

The work that follows was made possible by the contributions in time and effort by many people. In particular, thank you to many members of the lab both past (Phuong Le, Darian Wigfall, Simonne Francis, Valentina Ghisays) and present (Risha Shah) for assistance with oocyte preparations, molecular biology, and general technical assistance.

I would also like to extend a great many thanks to Dr. Colin Nichols. I consider myself extremely lucky to have found a lab which was both willing and unafraid to give an inexperienced freshman undergraduate the opportunity and latitude to develop as a scientist and as an independent and critical thinker. In the Nichols Lab, my productivity and growth as a scientist were limited only by my own time, dedication, ingenuity, and curiosity; and for this I am extraordinarily grateful.

Finally, I would like to express my deepest gratitude to Dr. Monica Sala-Rabanal. Not only do I appreciate your keeping me from burning the lab down in my early days; I can also say without any equivocation that I would not be where I am today without your patient and dedicated mentorship. It has been said that if you love what you do, you will never work a day in your life. To Monica, thank you for making that a reality for me these past four years.

Major funding was provided by NIH grants HL54171 and NS065201.

## TABLE OF CONTENTS

ACKNOWLEDGEMENTS	2
TABLE OF CONTENTS	3
LIST OF TABLES AND FIGURES	4
ABSTRACT	5
GENERAL INTRODUCTION	7
MATERIALS AND METHODS	13
PART I: POLYAMINE TRANSPORT BY POLYSPECIFIC OCT1, OCT2, AND OCT3	
I.    Introduction	18
II.   Results	18
III.  Discussion	25
PART II: MOLECULAR DETERMINANTS OF OCT3 SUBSTRATE SELECTIVITY	
I.    Introduction	31
II.   Results	34
III.  Discussion	43
REFERENCES	47

## LIST OF TABLES AND FIGURES

Table 1. Oligonucleotide Primers for Mutagenesis of rOCT3 and hOCT3	16
Table 2. Kinetics of MPP <sup>+</sup> and Spermidine Transport in OCT-Expressing Oocytes	21
Table 3. Mutations in rOCT3 and hOCT3 Examined in this Study	35
Figure 1. Tissue Distribution, Drug Clearance, and Predicted Topology of OCTs	8
Figure 2. Proposed Transport Mechanism of Mammalian OCTs	10
Figure 3. Compounds Examined in this Study	19
Figure 4. Transport of MPP <sup>+</sup> and Spermidine into <i>Xenopus laevis</i> Oocytes	20
Figure 5. Effect of Model Cations, Polyamines, and Polyamine Analogs on MPP <sup>+</sup> Transport	24
Figure 6. Inward- and Outward- Facing Homology Models of rOCT1 Tertiary Structure	32
Figure 7. Sequence Alignment of OCT1, OCT2, and OCT3	33
Figure 8. Transport of MPP <sup>+</sup> by rOCT3 and hOCT3 Mutants	36
Figure 9. Effect of Mutations in Rat OCT3 on Sensitivity to Model Cations, Polyamines, and Polyamine Analogs	39
Figure 10. Effect of Mutations in Human OCT3 on Sensitivity to Model Cations, Polyamines, and Polyamine Analogs	41

## ABSTRACT

Organic Cation Transporters (OCTs) are polyspecific, facilitative transporters of the Solute Carrier (SLC) family of membrane transport proteins that play major physiological roles in metabolite and drug clearance. OCT1 (*SLC22A1*) and OCT2 (*SLC22A2*) are expressed in hepatocytes and proximal tubule cells, whereas OCT3 (*SLC22A3*) is expressed in astrocytes. OCTs are promising drug targets, and thus elucidating the molecular mechanisms of substrate recognition in OCTs is crucial for rational drug design. While small, monovalent OCT substrates have been well-characterized, the ability of OCTs to transport or interact with larger, multiply charged cations remains largely unexplored. *Xenopus laevis* oocytes expressing mammalian OCTs were used to assess transport of polyamines, ubiquitous polyvalent cations of broad physiological and pharmacological import, but for which transport mechanisms in eukaryotic cells are unknown. Comparative dose-response analysis of radiolabelled substrate uptake revealed that the naturally occurring, trivalent polyamine spermidine is transported with lower affinity, but at similarly high turnover, than the model substrate methyl-4-phenylpyridinium (MPP<sup>+</sup>). To further investigate substrate specificity in OCTs, polyamine analogs of varying charge and hydrophobic character were screened for competition against MPP<sup>+</sup>: hydrophobicity is a principal requirement for polycationic substrate recognition, and OCT3 exhibits significantly higher hydrophobicity requirements than either of the two other isoforms. A hydrophobic cleft potentially capable of accommodating a variety of structures has been identified in the OCT structure by homology modelling of OCT1. In OCT3, replacement of an absolutely conserved residue within this pocket, D475 (TM11), by charge reversal (D475R), charge neutralization (D475N), or even charge replacement (D475E), completely abolishes MPP<sup>+</sup> uptake, suggesting it to be obligatory for OCT3-mediated transport, potentially by stabilization of positive charges within the putative

substrate binding pocket. Mutations at residues which line the putative binding pocket that are not conserved in OCT3 to the corresponding residue in OCT1 – L160F (TM2), F222Y (TM4), F447L (TM10), and E448Q (TM10) – generally decrease MPP<sup>+</sup> uptake, and also partially recapitulate the pharmacological selectivity profile of OCT1: Thus, interactions of naturally occurring polyamines, putrescine, spermidine, spermine, and potent OCT1 blockers and polyamine analogs, 1,10-diaminodecane (DA-10), decamethonium (TMA-10), bis-triethylaminodecane (TEA-10), and 1,10-bis-quinuclidinedecane (Quin-10) with wild-type OCT3 are weak, but are significantly potentiated in mutant OCT3. These results suggest that substrate specificity in OCTs is determined, structurally, at the level of the substrate-transporter interactions at the putative hydrophobic cleft of the protein, and that the particular residues identified above are key contributors in determining substrate affinity and/or sensitivity in OCTs.

## GENERAL INTRODUCTION

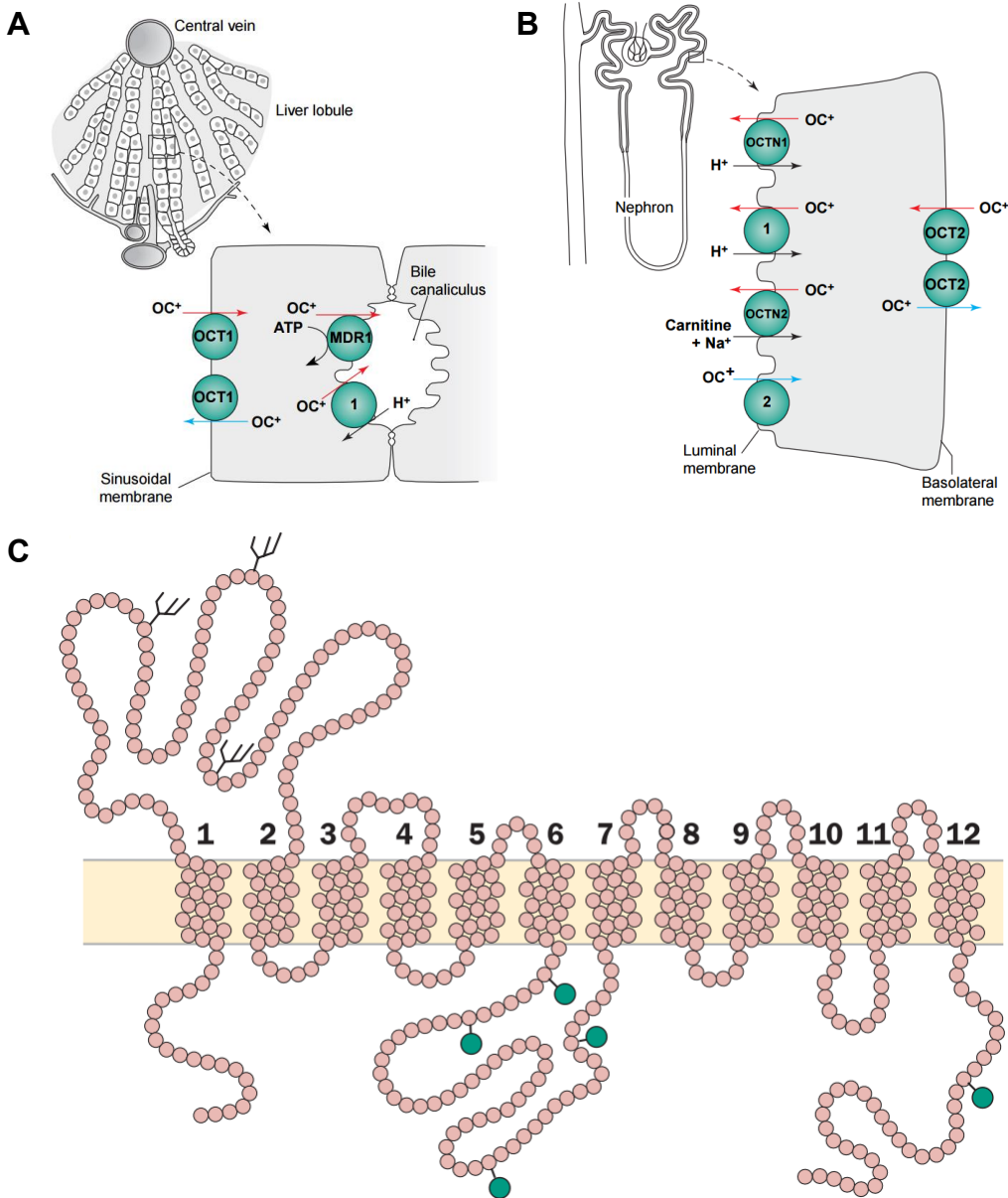
### *OCT Tissue Distribution and Physiological Significance*

Organic Cation Transporters (OCTs) belong to the *SLC22* family of facilitative transporters, and are typically localized to the basolateral membrane of polarized barrier epithelia, where they mediate the uptake, distribution, and efflux of cationic metabolites and drugs (Nies, Koepsell et al. 2011). In humans, OCT1 (*SLC22A1*) and OCT2 (*SLC22A2*) are predominantly expressed in the hepatocytes and proximal tubular cells of the liver and kidney, respectively (Gorboulev, Ulzheimer et al. 1997, Nishimura and Naito 2005), whereas OCT3 (*SLC22A3*) exhibits a broader tissue distribution, and is found in astrocytes (Busch, Karbach et al. 1998, Inazu, Takeda et al. 2003), the blood-cerebrospinal fluid barrier in choroid plexus epithelial cells (Hosoya and Tachikawa 2011), as well as in placenta, bronchial and intestinal epithelium (Lips, Volk et al. 2005, Muller, Lips et al. 2005, Sata, Ohtani et al. 2005). OCTs play major roles in drug clearance for both endogenous and xenobiotic compounds. In the liver, secretion of organic cations ( $OC^+$ ) into bile canaliculi is performed by OCT1 in the sinusoidal membrane in the biliary membrane. In the kidney,  $OC^+$  can be reabsorbed in proximal tubule cells by OCT3 (Figure 1A-1B) (Koepsell 2004).

### *Predicted OCT Topology*

All members of the *SLC22* transporter family, including the OCTs, have a similar predicted membrane topology which consists of 12 alpha-helical transmembrane (TM) domains with a large extracellular loop between TM1 and TM2 which is glycosylated (Keller, Egenberger et al. 2011, Brast, Grabner et al. 2012) and a large intracellular loop between TM6 and TM7 which is involved in post-transcriptional regulation (Figure 1C) (Koepsell, Lips et al. 2007).





**Figure 1. Tissue Distribution, Drug Clearance, and Predicted Topology of OCTs.**

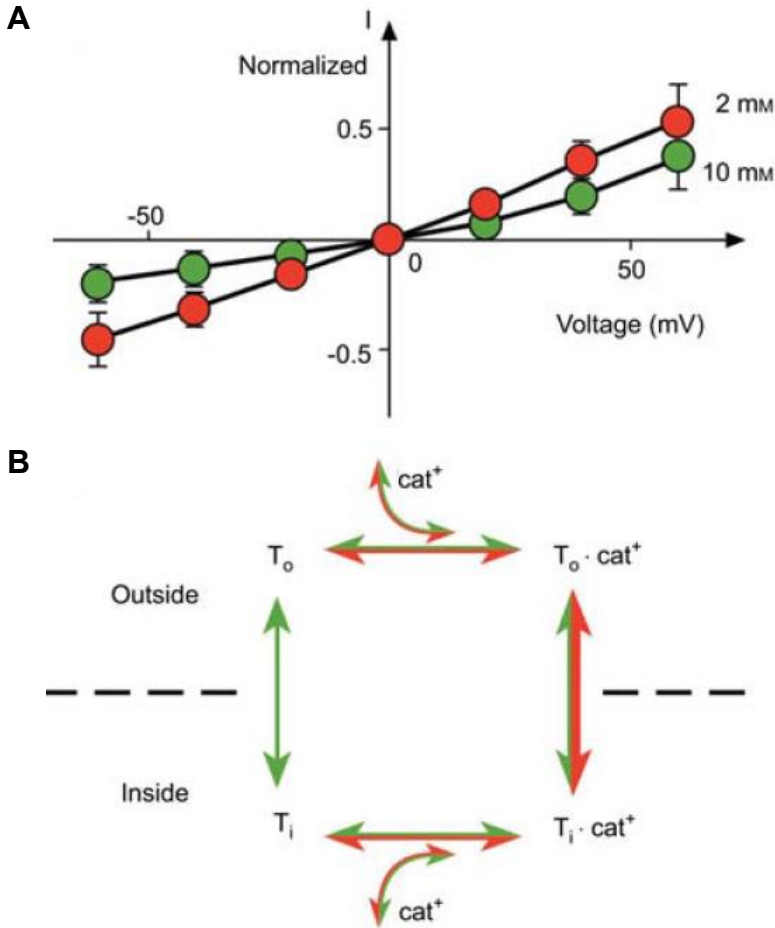
**A.** In the liver, OCT1 secretes organic cations (OC<sup>+</sup>) into bile canaliculi (red arrows) in the sinusoidal membrane coupled with multidrug resistance protein 1 (MDR1) and a H<sup>+</sup>-cation antiporter (1) in the biliary membrane. OCT1 also mediates translocation of OC<sup>+</sup> out of hepatocytes into sinusoids (blue arrows) **B.** In the kidney, filtered OC<sup>+</sup> drugs could be reabsorbed in proximal tubules through OCT2 (blue arrows). **C.** Predicted topology of human OCT1. Predicted glycosylation sites on the large extracellular loop (ψ) and predicted phosphorylation sites (green) are indicated. Adapted from (Koepsell, Lips et al. 2007).

### *Proposed OCT Transport Mechanism*

OCT-mediated facilitative diffusion has been demonstrated to be bidirectional in rOCT1 and rOCT2 (Koepsell 2011). In particular, electrophysiological experiments from giant patches in oocytes expressing rOCT2 have yielded similar apparent affinity constant ( $K_{0.5}$ ) values for the influx and efflux of choline and TEA at 0 mV holding potential, and have demonstrated that both inward and outward currents are voltage-dependent and symmetrical around the reversal potential (Figure 2A) (Budiman, Bamberg et al. 2000). These results are consistent with an alternating access model of transport which predicts electrogenic uptake as a result of substrate binding to the outward facing conformation, translocation of the bound substrate to the intracellular face which proceeds through an occluded state, dissociation of the substrate, and reorientation of the unbound substrate site to the extracellular face (Figure 2B) (Keller, Elfeber et al. 2005, Schmitt and Koepsell 2005). Additionally, OCTs have been demonstrated to translocate organic cations electrogenically in rat transporters OCT1, OCT2, and OCT3, and in the human transporters OCT1 and OCT2 (Gorboulev, Ulzheimer et al. 1997, Okuda, Urakami et al. 1999, Dresser, Gray et al. 2000). OCT mediated transport is independent of both  $\text{Na}^+$  and proton gradients (Busch, Quester et al. 1996, Koepsell, Lips et al. 2007) and is bidirectional, with efflux having been demonstrated for rat and human isoforms of OCT1, OCT2, and OCT3 (Nagel, Volk et al. 1997).

### *OCTs are Highly Polyspecific Cation Transporters*

OCTs interact with many, structurally very diverse monovalent cations (Wright 2005, Hendrickx, Johansson et al. 2013), including the model substrates tetraethylammonium (TEA) and the neurotoxin methyl 4-phenylpyridinium ( $\text{MPP}^+$ ). The relative molecular mass of compounds that have been shown to be transported by OCTs is less than around 500 with the smallest diameter of



**Figure 2. Proposed Transport Mechanism of Mammalian OCTs.**

**A.** Current-voltage relationship from giant inside-out patches of *Xenopus laevis* oocytes expressing rOCT2 demonstrating symmetric electrogenic transport of choline. **B.** Alternating access model of transport, illustrating individual transition steps involved in electrogenic cation transport. T<sub>o</sub> and T<sub>i</sub> states represent outward- and inward-facing conformations with the substrate binding site bound or unbound to a monovalent cation (cat<sup>+</sup>) substrate.

the molecules being below 4 Å (Schmitt and Koepsell 2005). Endogenous substrates include the metabolites choline and guanidine, and monoamine neurotransmitters such as the catecholamines (Koepsell, Lips et al. 2007).

Additionally, OCTs have been shown to recognize a broad range of pharmacologically active compounds (Nies, Koepsell et al. 2011), and polymorphisms that modify OCT function have been found to alter the response of patients to certain drugs, such as metformin (Choi and Song 2008, Zolk 2012). Their broad substrate selectivity makes the OCTs good candidates for rational drug design, but it can also result in undesired side effects, such as accumulation and toxicity in non-target organs, as well as competitive drug interactions (Ciarimboli 2011). Pharmacological and computational studies have established that hydrophobicity is a principal determinant for substrate recognition by OCTs (Bednarczyk, Ekins et al. 2003, Suhre, Ekins et al. 2005, Ahlin, Karlsson et al. 2008, Zolk, Solbach et al. 2009). At least one positive charge is required for transport (Harlfinger, Fork et al. 2005), but it has been reported that OCTs are also capable of transporting dicationic compounds, including the short polyamine putrescine (Winter, Elmquist et al. 2011).

#### *Polyamine Transport in Mammalian Systems*

Polyamines are ubiquitous organic cations that are implicated in a broad range of physiological processes, including cell-cell interactions, signaling, cell proliferation and differentiation, and ion channel modulation (Pegg 2009). In particular, the naturally occurring cytoplasmic polyamines, such as spermidine and spermine, for example, are responsible for inward rectification in Kir (*KCNJ*) potassium channels (Nichols and Lopatin 1997, Kurata, Phillips et al. 2004, Loussouarn, Marton et al. 2005), and thus play an important role in shaping membrane potential responses.

Extracellular polyamines have multiple effects in the central nervous system, including complex effects on NMDAR-type of glutamate receptors, enhancing NMDAR currents by channel opening and reducing activity by open channel block (Rock and Macdonald 1995). Polyamine levels are highly regulated; exchange mechanisms are key to this regulation, and because polyamines are positively charged at physiological pH, carrier-mediated transport systems are necessary for effective membrane permeation. Polyamine transporters have been characterized in prokaryotic cells and in yeast (Aouida, Leduc et al. 2005, Igarashi and Kashiwagi 2010), and connexin hemi-gap junctions have been shown to translocate polyamines in amphibian oocytes (Sha, Romano et al. 1996, Enkvetchakul, Ebihara et al. 2003), but the molecular identity of specific polyamine carriers in mammalian cells is still unclear (Poulin, Casero et al. 2012).

## MATERIALS AND METHODS

### *Chemicals*

Methyl [<sup>3</sup>H] 4-phenylpyridinium ([<sup>3</sup>H]MPP<sup>+</sup>) iodide (specific activity, 85 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO), and [<sup>3</sup>H]spermidine trihydrochloride (specific activity, 15 Ci/mmol) was purchased from PerkinElmer (Boston, MA). The following test compounds were examined: methyl 4-phenylpyridinium (MPP<sup>+</sup>), tetramethylammonium (TMA), tetraethylammonium (TEA), putrescine (Put), spermidine (Spdn), spermine (Spmn), 1,7-diaminoheptane (DA-7), 1,10-diaminodecane (DA-10), 1,6 bis-trimethylaminohexane (TMA-6), decamethonium (TMA-10), decyltrimethylammonium (TMA-10a), 1,6 bis-triethylaminohexane (TEA-6), 1,10 bis-triethylaminodecane (TEA-10), bis-quinuclidinehexane (Quin-6), and 1,10 bis-quinuclidinedecane (Quin-10). Synthesis of novel polyamine analogs TMA-6, TEA-6, TEA-10, Quin-6, and Quin-10 was performed by Harley T. Kurata and Gregory R. Dake (University of British Columbia, Vancouver, CAN).

### *OCT Constructs*

Mouse OCT1 and OCT2 were generously provided by Valentin Gorboulev (University of Wuerzburg, Germany), and rat and human OCT3 was a gift from Vadivel Ganapathy (Georgia Health Sciences University, Augusta, GA). Mouse OCT1 and OCT2 (mOCT1 and mOCT2, in the pBluescript II-SK vector) were linearized with XhoI (New England Biolabs, Ipswich, MA) and transcribed *in vitro* using the T3 mMessage mMachine kit (Applied Biosystems, Foster City, CA). Rat OCT3 (rOCT3) and human OCT3 (hOCT3), both in the pSPORT vector, were linearized with NotI and BamHI (New England Biolabs), respectively, and transcribed using the T7 mMessage mMachine kit (Applied Biosystems).

### *Heterologous Expression of OCTs in Oocytes*

Mature female *Xenopus laevis* frogs were purchased from Xenopus Express (Brooksville, FL). All animal protocols followed guidelines approved by the Washington University School of Medicine and the National Institutes of Health. Frogs were anaesthetized with 0.1% tricaine buffered with 0.1% NaHCO<sub>3</sub> prior to removal of a portion of the ovary, and were sacrificed by an overdose of tricaine. Stage V-VI oocytes were selected and maintained at 18 °C in modified Barth's solution containing 88 mM NaCl, 2.4 NaHCO<sub>3</sub>, 1 mM KCl, 0.3 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.4 mM CaCl<sub>2</sub>, 0.8 mM MgSO<sub>4</sub> and 10 mM Hepes/Tris (pH 7.4), and supplemented with 50 mg/l gentamicin, 6 mg/l ciprofloxacin, and 100 mg/l streptomycin sulphate/100,000 units/l penicillin G sodium (Life Technologies, Carlsbad, CA). Oocytes were injected one day after isolation with 25 ng OCT1, OCT2, or OCT3 cRNA, and maintained at 18 °C for 4–7 days. Experiments were carried out at 20–22 °C. In each experiment, non-injected oocytes from the same preparation served as controls.

### *Polyamine Transport Assay*

For dose-response experiments, oocytes were incubated in the presence of 0.1 μM to 1 mM MPP<sup>+</sup> (0.1 μM [<sup>3</sup>H]MPP<sup>+</sup>) or 0.1 μM to 10 mM spermidine (0.1 μM [<sup>3</sup>H]spermidine), in a buffer containing 100 mM NaCl, 3 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 10 mM Hepes/Tris (pH 7.4). After 30 min, oocytes were rinsed thoroughly with ice cold buffer, individually lysed with 2% sodium dodecyl sulfate, and assayed for radioactivity in a commercial scintillation cocktail (Econo-Safe, Research Products International, Mount Prospect, IL). Unless otherwise indicated, data are shown as the mean ± S.E. (standard error of the mean) of at least 3 experiments from different donor frogs, with 5–8 oocytes per condition.

### *Competition Assay*

The above assay procedure was also performed for competition studies. Oocytes were incubated in the presence of 0.1  $\mu\text{M}$  [ $^3\text{H}$ ]MPP $^+$  and 100  $\mu\text{M}$ , 1 mM, or 10 mM unlabeled test compound. In wild-type mOCT1, mOCT2, and rOCT3 expressing oocytes, [ $^3\text{H}$ ]MPP $^+$  uptake was measured against block by 1 mM and 10 mM of spermidine and spermine, and against block by 1 mM of TMA, TEA, and the polyamine analogs, DA-7, DA-10, TMA-6, TMA-10, TMA-10a, TEA-6, TEA-10, Quin-6, and Quin-10. In mutant rOCT3 and hOCT3 expressing oocytes, [ $^3\text{H}$ ]MPP $^+$  uptake was measured against the compounds, and also 100  $\mu\text{M}$  MPP $^+$ . Blocking potency or sensitivity were calculated as percentage of [ $^3\text{H}$ ]MPP $^+$  uptake in the absence of competing substrate.

### *Site-Directed Mutagenesis*

For generation of rat and human OCT3 mutants, PCR was performed using hOCT3 and rOCT3 wild-type cDNA as template and was carried out with the QuickChange Site-directed mutagenesis kit (Stratagene, Agilent Technologies, La Jolla, CA). A list of oligonucleotide primers used for mutagenesis, purchased from Sigma (St. Louis, MO), is shown in Table 1. Mutations were confirmed by DNA sequencing at the Protein and Nucleic Acid Chemistry Laboratory (PNAACL) at Washington University School of Medicine using Models 3130 and 3730 DNA sequencers (Applied Biosystems). Primer design and alignments of DNA sequence analysis were performed using DNASTAR Lasergene Suite 12.2 (DNASTAR, Madison, WI).



	Forward Primer (5'-3')	Reverse Primer (5'-3')
<b>rat OCT3</b>		
D473E	AGGCCTGTGT <b>GA</b> ATTTGGGGGGATCATAG	CTATGATCCCCCAA <b>ATC</b> ACACAGGCCT
D473N	AGGCCTGTGT <b>AA</b> CTTTGGGGGGATCATAG	CTATGATCCCCCAA <b>GTT</b> ACACAGGCCT
D473R	AGGCCTGTGT <b>CG</b> CTTTGGGGGGATCATAG	CTATGATCCCCCAA <b>GCG</b> ACACAGGCCT
L161F	CTTGGCTTC <b>TTT</b> GCTGGGGCTT	AAGCCCCAGC <b>AAA</b> GAAGCCAAG
F222Y	CTGGATGACTTG <b>TAT</b> GTGATTGTGACAG	CTGTCACAATCAC <b>ATAG</b> CAAGTCATCCAG
F445L	CCATGGCC <b>TTG</b> GAAATTGTTT	AAACAATTT <b>CAA</b> GGCCATGG
E446Q	CCATGGCCTT <b>CAA</b> ATTGTTT	AAACAAT <b>TTG</b> AAAGGCCATGG
F445L-E446Q	CCATGGCC <b>TTGCAA</b> ATTGTTT	AAACAAT <b>TTGCAA</b> GGCCATGG
<b>human OCT3</b>		
D478E	CAGGTCTGTGT <b>GAG</b> TTTGGGGGAATCATAGCC	GGCTATGATTCCCCCAA <b>CTC</b> ACACAGACCTG
D478N	CAGGTCTGTGT <b>AAT</b> TTTGGGGGAATCATAGCC	GGCTATGATTCCCCCAA <b>ATT</b> ACACAGACCTG
D478R	CAGGTCTGTGT <b>CGT</b> TTTGGGGGAATCATAGCC	GGCTATGATTCCCCCAA <b>ACG</b> ACACAGACCTG
L166F	TCCTGAAC <b>TTT</b> GGCTTCCTGACTGG	CCAGTCAGGAAGCC <b>AAA</b> GTTCAGGA
F226Y	CCTGCAAGGTGT <b>TAT</b> TGGAAAGGGGACGT	ACGTCCCCTT <b>CCATA</b> TACACCTGCAGG
F450L	CCATGGCC <b>TTG</b> GAAATTGTTT	AAACAATTT <b>CAA</b> GGCCATGG
E451Q	CCATGGCCTT <b>CAA</b> ATTGTTT	AAACAAT <b>TTG</b> AAAGGCCATGG
F450L-E451Q	CCATGGCC <b>TTGCAA</b> ATTGTTT	AAACAAT <b>TTGCAA</b> GGCCATGG

**Table 1. Oligonucleotide Primers for Mutagenesis of rOCT3 and hOCT3.**

Forward and reverse primers used for site-directed mutagenesis of rOCT3 and hOCT3. Amino acid codes for mutagenized residues are indicated in red.

### *Data Analysis*

SigmaPlot 10.0 (Systat Software, San Jose, CA), Accelrys Draw 4.1 (Accelrys Inc., San Diego, CA) and CorelDRAW X3 13.0 (Corel Corporation, Mountain View, CA) were used for curve fitting, statistics, and figure preparation. Kinetic parameters of transport were calculated with a non-linear regression, using (SigmaPlot 10.0). In non-injected oocytes, concentration-dependent MPP<sup>+</sup> and spermidine uptake values were modeled with a linear relationship. Data from OCT-expressing oocytes were best fitted to a Michaelis-Menten relationship plus a non-mediated component (Equation 1):

$$J = [J_{max} S / (K_{0.5} + S)] + K_d S \quad (1)$$

For which  $J$  is the uptake;  $S$  is the substrate concentration;  $J_{max}$  and  $K_{0.5}$  are, respectively, the derived maximum rate and the apparent affinity constant of carrier-mediated transport; and  $K_d$  is the rate of non-saturable, diffusive transport. Where indicated, paired Student's  $t$  test was applied to evaluate statistical differences between groups.

## PART I:

### POLYAMINE TRANSPORT BY POLYSPECIFIC OCT1, OCT2, AND OCT3

(Sala-Rabanal, Li et al. 2013)

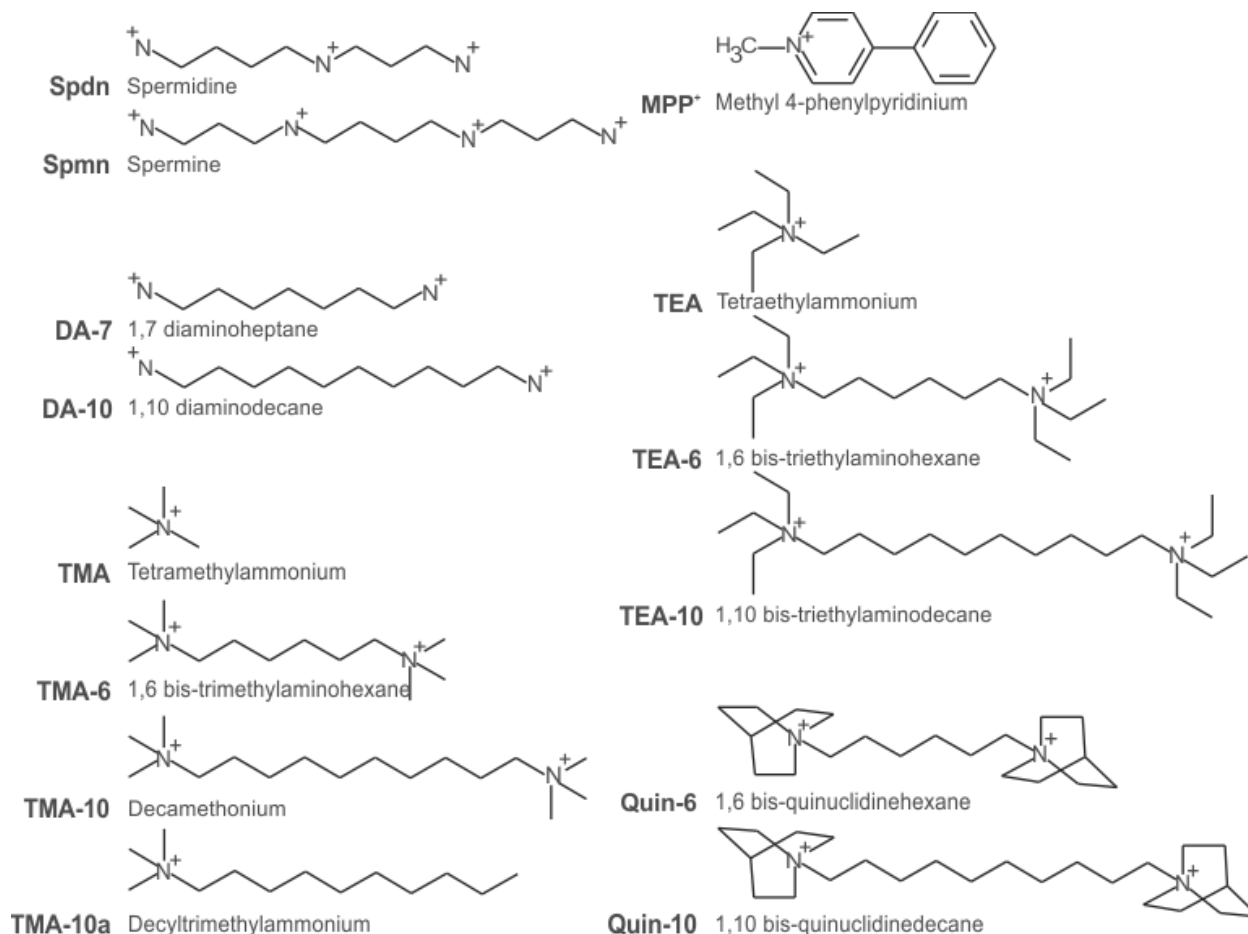
#### INTRODUCTION

The possibility that polyamines are substrates for organic cation transporters (OCTs) has received some attention (Busch, Quester et al. 1996, Winter, Elmquist et al. 2011), but the subject remains controversial (Grundemann, Hahne et al. 2003). In this part, *Xenopus* oocytes expressing mammalian OCT1, OCT2 or OCT3 were used to assess whether the natural longer-chain spermidine (Spdn, Figure 3) and spermine (Spmn), which carry net physiological charges of +3 or +4, respectively, are substrates for OCTs. To gain further insights into the contribution of the number and spatial organization of charges to substrate hydrophobicity, recognition and binding, the interactions between the transporters and an extended array of polyamine analogs differing in hydrophobic character, charge number, and distribution of charged groups were investigated. These results may prove useful in the refinement of existing pharmacophore models, towards the rational design of OCT-targeted biopharmaceutical compounds.

#### RESULTS

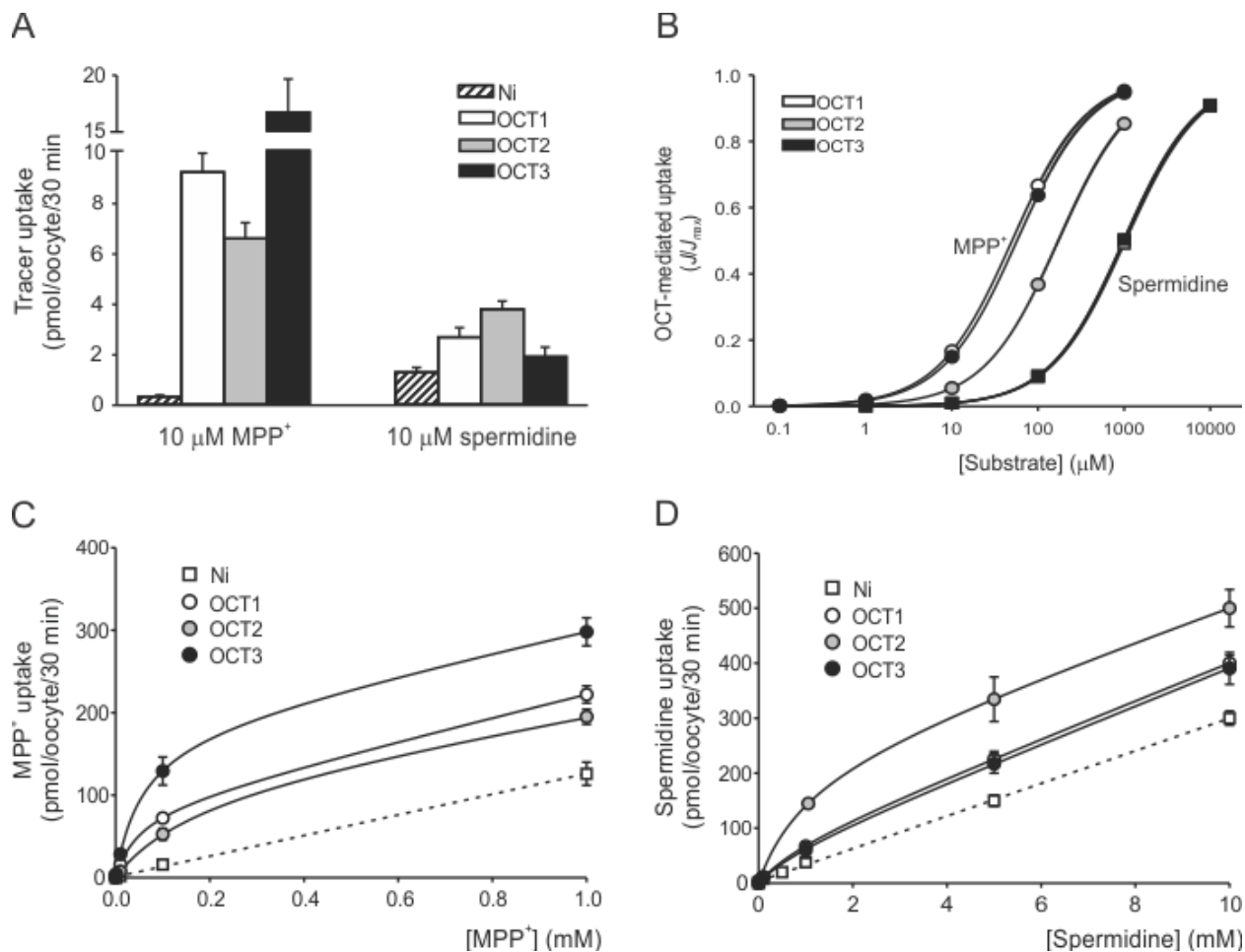
##### *OCT-Mediated MPP<sup>+</sup> and Spermidine Uptake*

The uptake of 10  $\mu\text{M}$  MPP<sup>+</sup> or 10  $\mu\text{M}$  spermidine (0.1  $\mu\text{M}$  [<sup>3</sup>H]MPP<sup>+</sup> or 0.1  $\mu\text{M}$  [<sup>3</sup>H]spermidine, respectively) into non-injected oocytes, and into oocytes injected with mouse OCT1 and OCT2 (mOCT1 and mOCT2) or rat OCT3 (rOCT3) cRNA, was assessed; results are summarized in Figure 4 and Table 2.



**Figure 3. Compounds Examined in this Study.**

Methyl 4-phenylpyridinium (MPP<sup>+</sup>) is a transported substrate for OCT1, OCT2, and OCT3. Tetramethylammonium (TMA) and tetraethylammonium (TEA) are model cations. Spermidine (Spdn) and spermine (Spmn) are naturally occurring polyamines. Polyamine analogs are classified according to the hydrophobicity of the end groups; within each class, compounds are ordered according to the number of carbons between end groups. Note that decylmethylammonium only has one charged group, but has been designated TMA-10a to indicate its structural resemblance to decamethonium, TMA-10.



**Figure 4. Transport of MPP<sup>+</sup> and Spermidine into *Xenopus laevis* Oocytes.**

Results are means  $\pm$  S.E. for 3 to 8 experiments from different oocyte preparations. **A.** Uptake of 10  $\mu$ M MPP<sup>+</sup> (0.1  $\mu$ M [<sup>3</sup>H]MPP<sup>+</sup>) or 10  $\mu$ M spermidine (0.1  $\mu$ M [<sup>3</sup>H]spermidine) in non-injected oocytes (Ni, hatched bars) and in oocytes expressing mOCT1 (white bars), mOCT2 (gray bars) or rOCT3 (black bars). **B-D.** Concentration dependence of MPP<sup>+</sup> and spermidine uptake into non-injected oocytes (squares) or into OCT-expressing oocytes (circles). Oocytes were incubated in presence of 0.1  $\mu$ M [<sup>3</sup>H]MPP<sup>+</sup> or 0.1  $\mu$ M [<sup>3</sup>H]spermidine and increasing concentrations of unlabeled substrate. Dashed lines, MPP<sup>+</sup> and spermidine uptake in non-injected oocytes was non-saturable, and followed a linear relationship ( $r^2 = 0.9994$  for both substrates) with estimated slopes of  $125 \pm 2$  nl/oocyte/30 min (MPP<sup>+</sup>) and  $30 \pm 1$  nl/oocyte/30 min (spermidine). Solid lines, uptake data from OCT-expressing oocytes were fitted to Equation 1 to estimate the kinetic parameters of OCT-mediated transport, plus a non-mediated component.

	$K_{0.5}$ ( $\mu\text{M}$ )	$J_{max}$ ( $\text{pmol}/\text{oocyte}/30 \text{ min}$ )	$K_d$ ( $\text{nl}/\text{oocyte}/30 \text{ min}$ )
<b><i>MPP<sup>+</sup></i></b>			
<b>OCT1</b>	$50 \pm 1$	$87 \pm 1$	$139 \pm 1$
<b>OCT2</b>	$172 \pm 19$	$117 \pm 20$	$95 \pm 14$
<b>OCT3</b>	$57 \pm 4$	$183 \pm 6$	$125 \pm 5$
<b><i>Spermidine</i></b>			
<b>OCT1</b>	$996 \pm 21$	$67 \pm 8$	$34 \pm 1$
<b>OCT2</b>	$1036 \pm 19$	$225 \pm 2$	$30 \pm 1$
<b>OCT3</b>	$983 \pm 36$	$54 \pm 11$	$34 \pm 1$

**Table 2. Kinetics of MPP<sup>+</sup> and Spermidine Transport in OCT-expressing Oocytes.**

Data are from the experiments shown in Figure 4B-D. Dose-response uptake values were fitted to Equation 1 to estimate the apparent affinity constants ( $K_{0.5}$ ) and highest rates ( $J_{max}$ ) of MPP<sup>+</sup> and spermidine carrier-dependent transport, plus a non-mediated, diffusive component ( $K_d$ ). The standard error of the fits is shown.

In non-injected oocytes, MPP<sup>+</sup> uptake was  $0.5 \pm 0.1$  pmol/oocyte/30 min, and spermidine uptake was  $2.0 \pm 0.3$  pmol/oocyte/30 min. In OCT-expressing oocytes, MPP<sup>+</sup> transport rates were 15– to 35–fold higher than in non-injected oocytes, whereas those for spermidine increased more modestly above the background, up to about 3–fold (Figure 4A). To ascertain the kinetic properties of OCT-mediated and non-specific transport, the concentration dependence of MPP<sup>+</sup> and spermidine uptake in the different experimental groups was investigated (Figure 4B-4D, Table 2).

In non-injected oocytes, MPP<sup>+</sup> (Figure 4C) and spermidine (Figure 4D) uptake was non-saturable, and the dose-response data followed a linear relationship ( $r^2 > 0.999$ ) with estimated slopes of  $125 \pm 2$  nl/oocyte/30 min (MPP<sup>+</sup>) and  $30 \pm 1$  nl/oocyte/30 min (spermidine). MPP<sup>+</sup> and spermidine uptake data from OCT-injected oocytes (Figure 4C-4D) were best fitted to Equation 1, that is, a Michaelis-Menten relationship with apparent affinity constants ( $K_{0.5}$ ) and maximum rates ( $J_{max}$ ) of carrier-mediated transport, plus a non-mediated component ( $K_d$ ). Spermidine was transported by the OCTs with approximately 6 to 20 fold lower affinity than MPP<sup>+</sup>; thus,  $K_{0.5}$  for MPP<sup>+</sup> was approximately 50  $\mu$ M in OCT1, 170  $\mu$ M in OCT2, and 60  $\mu$ M in OCT3, whereas for spermidine,  $K_{0.5}$  was 1 mM in OCT1, OCT2, and OCT3 (Table 2, Figure 4B). On the other hand,  $J_{max}$  values for MPP<sup>+</sup> and spermidine were within the same range (Table 2), indicating that the turnover rate of transport is similar for both compounds.  $K_d$  was estimated at roughly 100–140 nl/oocyte/30 min for MPP<sup>+</sup> and roughly 30–34 nl/oocyte/30 min for spermidine (Table 2), and this is consistent with the linear rate of MPP<sup>+</sup> and spermidine uptake in non-injected oocytes.

This suggests that, in *Xenopus* oocytes, MPP<sup>+</sup> and spermidine are taken up by endogenous mechanisms; because these uptake experiments were conducted in presence of 2 mM extracellular Ca<sup>2+</sup>, it is likely that these mechanisms are distinct from the Ca<sup>2+</sup>-sensitive connexin hemichannels described previously (Enkvetchakul, Ebihara et al. 2003). Interestingly, it has been observed that

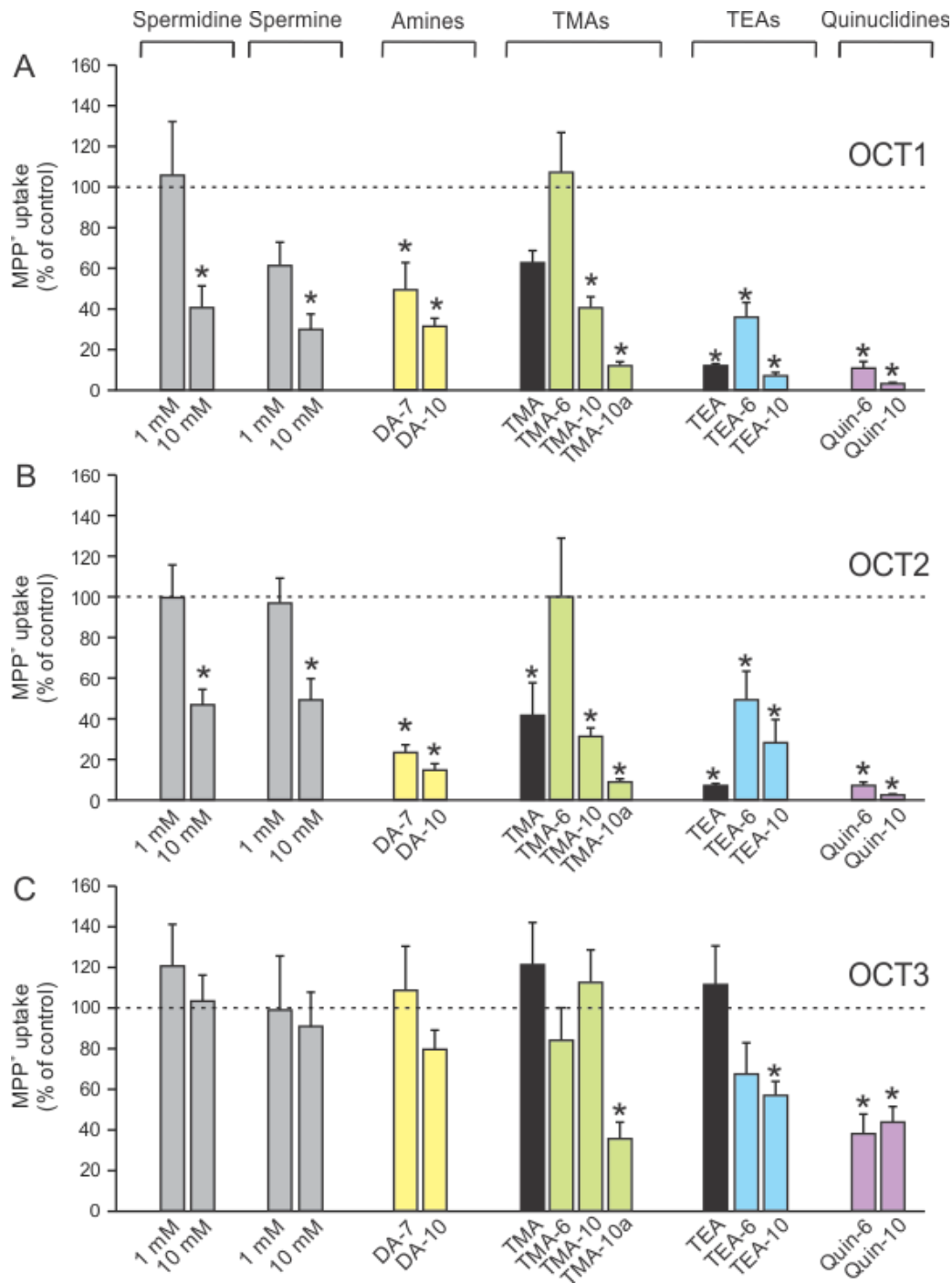
secondary and tertiary amines are transported across the oocyte membrane by a passive,  $\text{Ca}^{2+}$ -insensitive carrier (Burckhardt and Thelen 1995), but the molecular identity of this transport system remains elusive (Sobczak, Bangel-Ruland et al. 2010).

#### *Inhibition of MPP<sup>+</sup> Uptake by Polyamines and Polyamine Analogs*

To gain further insights into OCT substrate specificity, natural polyamines and a selection of structurally analogous chemicals (Figure 3) were screened for effect on the uptake of 0.1  $\mu\text{M}$  [<sup>3</sup>H]MPP<sup>+</sup> into oocytes expressing OCT1, OCT2 or OCT3. Spermidine and spermine were used at 1 and 10 mM, and the other compounds were tested at 1 mM (Figure 5). First, spermidine and spermine were compared with alkylamines that are equivalent in overall length but possessing only the two terminal charges, namely 1,7-diaminoheptane (DA-7) and 1,10-diaminodecane (DA-10). At 1 mM, spermidine had no effect on MPP<sup>+</sup> uptake into OCT1 (Figure 5A), or OCT2 (Figure 5B) oocytes, but at 10 mM it blocked approximately 60 and 55%. 1 mM spermine blocked only roughly 40% of the uptake in OCT1- (Figure 5A), and failed to inhibit uptake in OCT2-expressing oocytes (Figure 5B); at 10 mM, spermine blocked approximately 50% of the uptake in OCT1 and OCT2 oocytes. DA-7 and DA-10 inhibited around 50 and 70% of MPP<sup>+</sup> uptake in OCT1 (Figure 5A), and about 80% in OCT2 oocytes (Figure 5B). Spermidine, spermine, DA-7, and DA-10 did not have a significant blocking effect on MPP<sup>+</sup> uptake into OCT3-expressing oocytes.

Next, it was observed that increasing the hydrophobicity of the end charged group, and when more than one charge is present, the relative distance between them, improves recognition by the transporters. For example, in OCT1- and OCT2-expressing oocytes, MPP<sup>+</sup> uptake dropped roughly 40% and 60% in the presence of the monovalent cation TMA, but was unaffected in the presence of TMA-6 (Figure 5A-5B), in which two terminal trimethylamine groups are separated





**Figure 5. Effect of Model Cations, Polyamines, and Polyamine Analogs on MPP<sup>+</sup> Transport.**

Uptake of 0.1  $\mu\text{M}$  [<sup>3</sup>H]MPP<sup>+</sup> in oocytes expressing **A.** OCT1 **B.** OCT2 or **C.** OCT3 was measured in the absence or in the presence of TMA, TEA, natural polyamines (spermidine and spermine), and selected polyamine analogs. Spermidine and spermine were used at 1 and 10 mM, and the rest were tested at 1 mM. Uptake in non-injected oocytes was not affected by any test compound. \* $p < 0.05$  as compared to uptake in absence of external inhibitors (paired Student's  $t$  test).

by only a hexyl chain. In the presence of TMA-10, in which the same terminal groups are separated by a decyl chain, uptake decreased 60% in OCT1 (Figure 5A) and 70% in OCT2 (Figure 5B); TMA-10a, structurally analogous to TMA-10 but with only one charged group, was a more potent blocker in both isoforms than its divalent counterpart (Figure 5A-5B). TEA, which is a more hydrophobic compound than TMA, inhibited roughly 90% of the OCT1- and OCT2-mediated MPP<sup>+</sup> uptake; blocking potency was reduced, but still significant, for TEA-6, in which two end triethylamine groups are separated by a hexyl chain, and restored in TEA-10 (Figure 5A-5B), in which the two charges are more distant. In OCT1 and OCT2 oocytes, MPP<sup>+</sup> uptake was blocked over 90% in presence of quinuclidine analog Quin-6, and almost abolished in presence of the decyl species Quin-10 (Figure 5A-5B).

OCT3 appeared to be less tolerant than the other two isoforms: only monovalent TMA-10a, and highly hydrophobic divalents TEA-6, TEA-10, Quin-6 and Quin-10 inhibited the uptake of MPP<sup>+</sup> into OCT3-expressing oocytes (Figure 5C).

## DISCUSSION

### *Polyamines Are Substrates for the Organic Cation Transporters*

The naturally occurring polyamines putrescine (1,4-diaminobutane), spermidine, and spermine are present in all cells, where they play prominent roles in cell growth, proliferation and differentiation (Pegg 2009). Control of the cytosolic polyamine pool is critical for normal physiology, and alterations in polyamine homeostasis have been linked to stroke, renal failure and cancer (Casero and Pegg 2009). Specific exchangers, transporters and multimeric uptake systems critical to the regulation of polyamine content have been described in bacteria and yeast (Aouida, Leduc et al. 2005, Igarashi and Kashiwagi 2010). In higher organisms, connexin hemi-gap junctions mediate

Ca<sup>2+</sup>-sensitive exchange of spermidine in *Xenopus* oocytes (Enkvetchakul, Ebihara et al. 2003), and the diamine exporter complex SLC3-y<sup>+</sup>LAT has been shown to translocate putrescine and acetylpolyamine intermediaries in colon epithelial cells (Uemura, Yerushalmi et al. 2008); otherwise, specific polyamine carrier proteins have yet to be identified.

Here, the hypothesis that polyamines are substrates for the organic cation transporters, namely OCT1 (*SLC22A1*), OCT2 (*SLC22A2*) and OCT3 (*SLC22A3*) has been examined. Previously, it has been shown that putrescine and the polyamine precursor agmatine, both divalent at physiological pH, can be transported by human and rodent OCTs (Grundemann, Hahne et al. 2003, Winter, Elmquist et al. 2011). This observation has been extended to demonstrate that spermidine, which is longer and carries an additional positive charge, is also a transported substrate of OCT1, OCT2 and OCT3 (Figure 4, Table 2). Modest inward currents were induced in presence of 1 mM spermine in *Xenopus* oocytes expressing rOCT1 (Busch, Quester et al. 1996), suggesting that the longer, tetravalent polyamine is also transported, albeit at a low rate. Though mouse or rat isoforms were used in this study, and interspecies differences in transport efficiency have been reported for certain compounds (Dresser, Gray et al. 2000, Suhre, Ekins et al. 2005), it seems likely that spermidine will also be transported by human OCTs: residues that are essential for substrate specificity and binding in rodent OCTs are conserved in the respective human homologs (Gorboulev, Shatskaya et al. 2005, Popp, Gorboulev et al. 2005, Koepsell 2011).

#### *Physiological and Pharmacological Significance of OCT-Mediated Polyamine Transport*

The identification of OCTs as relevant polyamine exchange systems may contribute to further the understanding of the physiological roles of polyamines, and the mechanisms involved in intracellular polyamine regulation. In general, circulating polyamine levels are low; for example,

the concentration of spermidine in blood is in the range of 300 nM (Van Dobbenburgh, Houwen et al. 1983). Here it has been shown that the  $K_{0.5}$  for OCT-mediated spermidine uptake is approximately 1 mM (Table 2), and thus the OCTs may not play a significant role in cellular polyamine uptake under physiological conditions. However, intracellular polyamine concentrations in certain tissues, such as the glia, are in the mM range (Benedikt, Inyushin et al. 2012), and in these cells the OCTs could be implicated in polyamine efflux.

Voltage-dependent block by cytosolic polyamines is the major process underlying inward rectification in Kir channels (Nichols and Lopatin 1997), which control membrane potential and potassium homeostasis in many tissues and organs, including the central nervous system. Human and rodent OCT2 and OCT3 are expressed in neurons of various areas of the brain (Busch, Karbach et al. 1998) and in glial cells (Inazu, Takeda et al. 2003, Cui, Aras et al. 2009), respectively, where they may participate in polyamine-mediated and Kir channel modulation and cell-to-cell communication (Kucheryavykh, Shuba et al. 2008, Benedikt, Inyushin et al. 2012). On the other hand, loss-of-function, overactive, or deregulated Kir channels result in disorders ranging from deafness, epilepsy and autism, to the systemic Andersen-Tawil, Barter and EAST/SeSAME syndromes (Bockenbauer, Feather et al. 2009, Scholl, Choi et al. 2009, Hibino, Inanobe et al. 2010, Inyushin, Kucheryavykh et al. 2010, Sala-Rabanal, Kucheryavykh et al. 2010). Use of OCTs as a vehicle to deliver high-affinity polyamine-like compounds to help alleviate some of these conditions is a potentially powerful pharmacological tool.

Conversely, since OCTs are expressed in organs of drug absorption, disposition and excretion, such as the intestinal, liver, and kidney epithelia (Nies, Koepsell et al. 2011), they might be exploited to enhance the bioavailability of pharmaceuticals aimed to diagnose or treat diseases caused by polyamine imbalance. Carcinogenesis and tumor growth have been associated with

increased intracellular polyamine levels (Casero and Marton 2007), and thus OCTs might be targeted for the delivery of cytotoxic polyamine analogs or polyamine-conjugated imaging probes. This may aid particularly in the management of neuropathies and brain tumors: OCT1 and OCT2 have been shown to express in rodent and human brain microvascular epithelium (Lin, Tai et al. 2010), and have been hypothesized to mediate the blood-to-brain influx of agmatine (Winter, Elmquist et al. 2011), a neuromodulator which also has potent antiproliferative properties (Wang, Su et al. 2005, Isome, Lortie et al. 2007). OCT3 is found in the apical membrane of choroid plexus epithelial cells where it has been shown to participate in the cerebrospinal fluid-to-blood clearance of neurotoxic metabolites (Hosoya and Tachikawa 2011), and might thus be considered as a pathway for the elimination of polyamine drug byproducts.

#### *Spermidine is a Low-Affinity Substrate for OCTs: Insights into OCT Substrate Specificity*

These results are consistent with, and complementary to, current understanding of OCT substrate selectivity. OCTs are known to interact with virtually all natural or xenobiotic primary, secondary, tertiary or quaternary amines, with  $K_{0.5}$ ,  $K_i$  or  $IC_{50}$  values in the  $\mu\text{M}$  (high affinity) to  $\text{mM}$  (low affinity) range (Koepsell, Lips et al. 2007). It has been established that the presence of one positive charge is essential for substrate translocation by OCTs (Harlfinger, Fork et al. 2005), and one absolutely conserved residue within the substrate binding site (D475) is implicated in the ion-pair interaction that purportedly initiates substrate binding (Gorboulev, Volk et al. 1999, Gorboulev, Shatskaya et al. 2005, Popp, Gorboulev et al. 2005). Though the vast majority of known OCT substrates or blockers are monovalent cations, OCTs can also transport compounds that carry a net physiological charge of +2, as putrescine (Winter, Elmquist et al. 2011); +3, as seen here for spermidine; and perhaps even +4, as has been suggested for spermine (Busch, Quester et al. 1996).

Here, it has been shown that spermidine is a substrate for OCT1, OCT2 and OCT3, but it is transported with low efficiency, i.e. at a similar turnover rate than the model substrate MPP<sup>+</sup>, but with up to 20-fold lower apparent affinity (Figure 4 and Table 2). While the  $K_{0.5}$  for OCT-mediated spermidine transport was estimated at approximately 1 mM (Table 2), 50% inhibition of MPP<sup>+</sup> uptake was observed in OCT1 and OCT2-expressing oocytes only in presence of 10 mM spermidine (Figure 5B), and neither 1 mM nor 10 mM spermidine had any blocking effect in OCT3-expressing oocytes (Figure 5C). Based on mutagenesis, pharmacology and homology modeling data, it has been hypothesized that OCTs contain distinct, but partially overlapping, binding sites for different substrates (Gorboulev, Volk et al. 1999, Gorboulev, Shatskaya et al. 2005, Koepsell 2011). When administered together, cations bound to high-affinity sites can partially or totally compete away cations bound to low-affinity sites (Gorbunov, Gorboulev et al. 2008), and this may help explain the apparent discrepancy between the above dose-response data and competition assay results.

On the other hand, the low apparent affinity of OCTs for the polyamines may result from the relative hydrophilic nature of those charges, which in effect reduce the overall hydrophobicity of the compound, rather than the multiple charges carried by spermidine or spermine. Unlike spermidine or spermine, the diamines DA-7 and DA-10, which are significantly more hydrophobic than the polyamines (Weiger, Langer et al. 1998), effectively inhibited MPP<sup>+</sup> uptake in OCT1- and OCT2-expressing oocytes. Among the different dicationic analogs tested, the presence of more hydrophobic terminal groups generally resulted in a stronger block; within each class, elongating the aliphatic chain between charges seemed to improve recognition. For example, TMAs were overall weaker blockers than TEAs or quinuclidines, and among TMAs, TMA-10 and TMA-10a were stronger than TMA-6.

Several previous studies have shown that there is a direct correlation between the hydrophobic character of a given compound and the strength of its interaction with the OCTs (Bednarczyk, Ekins et al. 2003, Suhre, Ekins et al. 2005, Ahlin, Karlsson et al. 2008, Zolk, Solbach et al. 2009). In particular, it has been described that increasing the length of the alkyl chain in *n*-tetraalkylammonium compounds, which effectively increases their hydrophobicity, translates into a proportional increase in the  $IC_{50}$  values for inhibition of hOCT1-mediated radiolabelled TEA uptake in mammalian cells (Zhang, Gorset et al. 1999, Bednarczyk, Ekins et al. 2003), and observed that TEA was a more effective blocker of MPP<sup>+</sup> uptake than TMA in OCT1- and OCT2-expressing oocytes (Figure 5A and 5B). Whereas very hydrophobic, cyclic dicationic chemicals such as the broad-range herbicide paraquat (Chen, Zhang et al. 2007), and the antiparasitic drugs pentamidine and furamidine (Ming, Ju et al. 2009), are high-affinity substrates or inhibitors of OCTs, the more hydrophilic diamines putrescine and agmatine are transported with very low efficiency (Winter, Elmquist et al. 2011).

Though the relationship between hydrophobicity and binding affinity of OCT substrates and inhibitors with multiple charges requires further investigation, these data suggest that, as with their monovalent counterparts, recognition and binding of divalent substrates depend largely on their overall hydrophobic character.

## PART II:

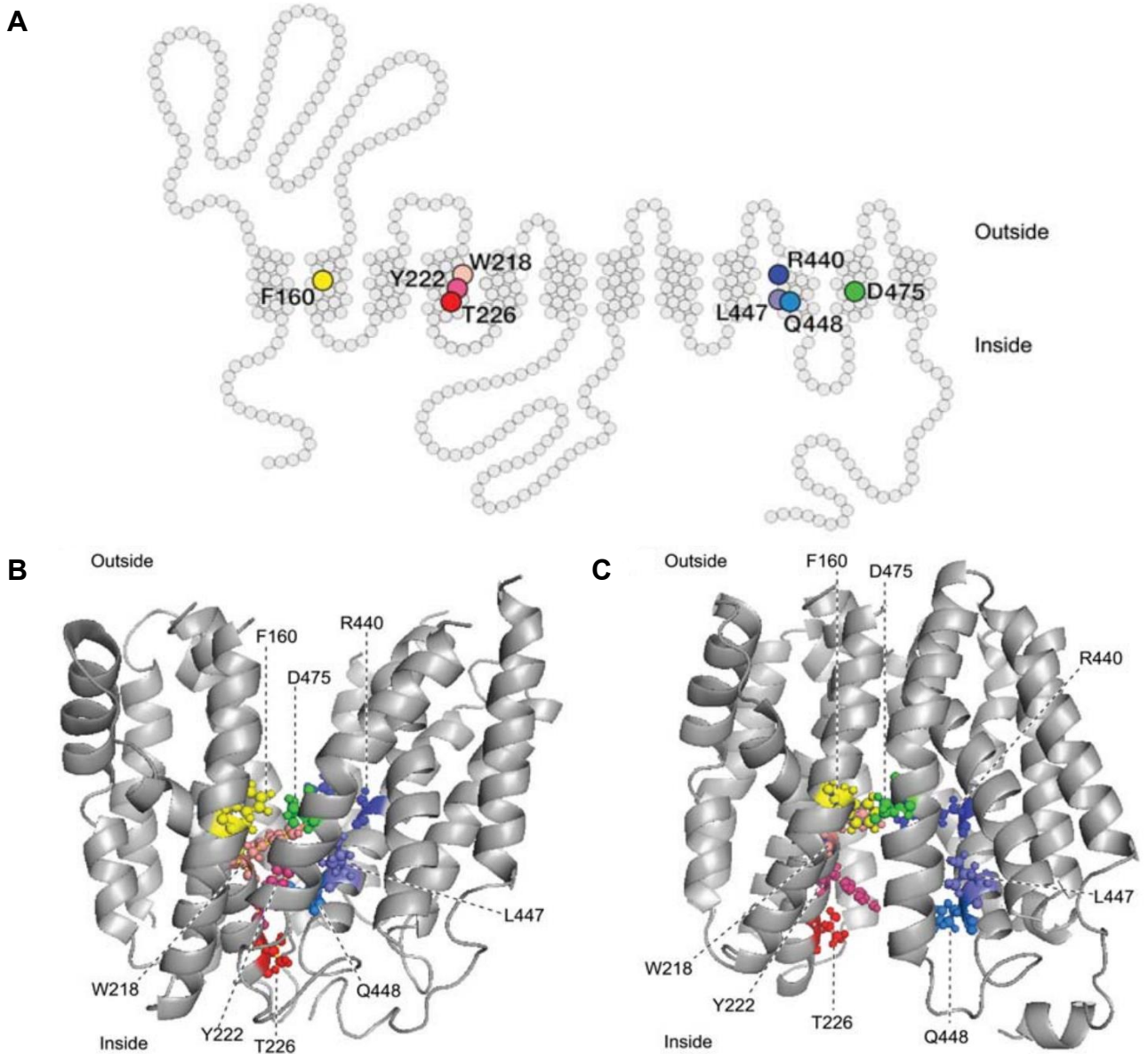
### MOLECULAR DETERMINANTS OF OCT3 SUBSTRATE SELECTIVITY

(Li, Nichols et al. 2015)

#### INTRODUCTION

Part I demonstrated that OCT substrates interact less efficiently with OCT3 than with OCT1 or OCT2, with only the most hydrophobic compounds inhibiting MPP<sup>+</sup> uptake (Sala-Rabanal, Li et al. 2013), indicating that OCT3 has more stringent binding requirements than OCT1 or OCT2 which may respond to differences in sequence and structural organization. No three-dimensional structure of OCTs has yet been solved, but homology models of OCT1 and OCT2, based on biochemical data and high-resolution crystal structures of *E. coli* LacY and GlpT (Abramson, Smirnova et al. 2003, Huang, Lemieux et al. 2003, Higashi, Imamura et al. 2014), suggest that seven of the predicted twelve transmembrane helices (TM) of OCTs, namely TMs 1, 2, 4, 5, 7, 10 and 11, fold in a large hydrophobic cleft capable of accommodating a wide variety of chemical species (Popp, Gorboulev et al. 2005, Zhang, Shirahatti et al. 2005). OCT1 and OCT2 share 70% sequence identity and have similar substrate selectivity profiles (see Figure 5 for example), reflected in the relatively similar makeup of their predicted binding pockets (Wright and Dantzer 2004, Koepsell, Lips et al. 2007, Koepsell 2011). OCT3 orthologs share only 50% sequence identity with OCT1 or OCT2, which may translate into architectural differences substantial enough to impact transport or inhibition kinetics and may account for their apparently tighter binding constraints. In this part, residues implicated as being crucial for affinity and/or selectivity in the rOCT1 homology model based on LacY (Figure 6) and which are not conserved in either rat or human OCT3 (Figure 7), are mutagenized to their corresponding rOCT1 residues to investigate





**Figure 6. Inward- and Outward- Facing Homology Models of rOCT1 Tertiary Structure.**

Structural models of rOCT1 with highlighted amino acids that are crucial for substrate affinity as identified by mutagenesis experiments for uptake of TEA and MPP<sup>+</sup>. **A.** Predicted membrane topology of rOCT1. **B.** Inward-facing conformation was accomplished by using the crystal structures of inward-facing conformations of lactose permease LacY from *E. coli* (Abramson, Smirnova et al. 2003, Popp, Gorboulev et al. 2005). **C.** Outward-facing conformation was modeled by employing a putative rearrangement mechanism for lactose transport by LacY using biochemical data (Schmitt and Koepsell 2005).

	140	<b>TM2</b>			
rOCT1	...	VCGDAWKVDL	FQSCVNLGF <b>F</b>	LGSLVVGZIA	DRFGRKLCLL...
mOCT1	...	VCGDAWKVDL	FQSCVNLGF <b>F</b>	LGSLVVGZIA	DRFGRKLCLL...
mOCT2	...	VCAHSWMLDL	FQSLVNVGF <b>F</b>	IGAVGIGYLA	DRFGRKFCLL...
rOCT3	...	VCGNAWMLDL	TQAILNLGFL	AGAFTLGYAA	DRYGR LIVYL...
hOCT3	...	VCVNAWMLDL	TQAILNLGFL	TGAFTLGYAA	DRYGRIV IYL...
	200	<b>TM4</b>			
rOCT1	...	TSMLLFRLLO	GMVSKGS <b>W</b> VS	GYTLIT <b>T</b> EFVG	SGYRRTTAIL...
mOCT1	...	TSMLLFRLLO	GMVSKGS <b>W</b> VS	GYTLIT <b>T</b> EFVG	SGYRRTTAIL...
mOCT2	...	AWMLVFRFLQ	GLVSKAG <b>W</b> LI	GYILIT <b>T</b> EFVG	LGYRRTVGIC...
rOCT3	...	SVFVIFRFLQ	GVEFGKAW <b>M</b> T	CFVIV <b>T</b> EIVG	SKQRRIVGIV...
hOCT3	...	PVFVIFRFLQ	GVEFGK <b>T</b> W <b>M</b> T	CFVIV <b>T</b> EIVG	SKQRRIVGIV...
	420	<b>TM10</b>			
rOCT1	...	MIFIPHELHW	LNVTLACL <b>G</b> R	MGATIV <b>L</b> Q <b>M</b> V	CLVNAELYPT...
mOCT1	...	MIFIPHELHW	LNVTLACL <b>G</b> R	MGATIV <b>L</b> Q <b>M</b> V	CLVNAELYPT...
mOCT2	...	SVFIPDDLQW	LKITVACL <b>G</b> R	MGATIA <b>Y</b> E <b>M</b> V	CLVNAELYPT...
rOCT3	...	TAFLPEGIPW	LRTTVATL <b>G</b> R	LGITMA <b>F</b> E <b>I</b> V	YLVNSELYPT...
hOCT3	...	TAFLPEGIAW	LRTTVATL <b>G</b> R	LGITMA <b>F</b> E <b>I</b> V	YLVNSELYPT...
	460	<b>TM11</b>			
rOCT1	...	FIRNLGMMVC	SALC <b>D</b> LGGIF	TPFMVFR LME	VWQALPLILF...
mOCT1	...	FIRNLGMMVC	SALC <b>D</b> LGGIF	TPFMVFR LME	VWQALPLILF...
mOCT2	...	YIRNLAVLVC	SSMC <b>D</b> IGGIV	TPFLVYRLTD	IWLEFPLVVF...
rOCT3	...	TLRNFGVSLC	SGLC <b>D</b> FGGII	APFLLFRLAA	IWLELPLIIF...
hOCT3	...	TLRNFGVSLC	SGLC <b>D</b> FGGII	APFLLFRLAA	VWLELPLIIF...

### Figure 7. Sequence Alignment of OCT1, OCT2, and OCT3.

Highlighted residues are those which have been identified by mutagenesis and rOCT1 homology modelling as potentially important for determining substrate affinity and/or sensitivity. Blue highlighting indicates hydrophobic pocket residues at these positions conserved compared to rOCT1. Yellow highlighting indicates hydrophobic pocket residues at these positions which are not conserved compared to rOCT1. Pink highlighting indicates an absolutely conserved aspartate residue which has been proposed as the cationic binding site in the transporter binding pocket.

the role of these residues in the differential substrate selectivity profiles of OCT3 and either OCT1 or OCT2 (Table 3). Additionally, an aspartate residue in rOCT1 (D475) has been proposed as the cationic binding site in OCT1 and OCT2 and is conserved in OCT3. Here, the role of this residue in rOCT3 (D473) and hOCT3 (D478) is also investigated (Table 3).

## RESULTS

### *MPP<sup>+</sup> Uptake by OCT3 Substrate Binding Site Mutants*

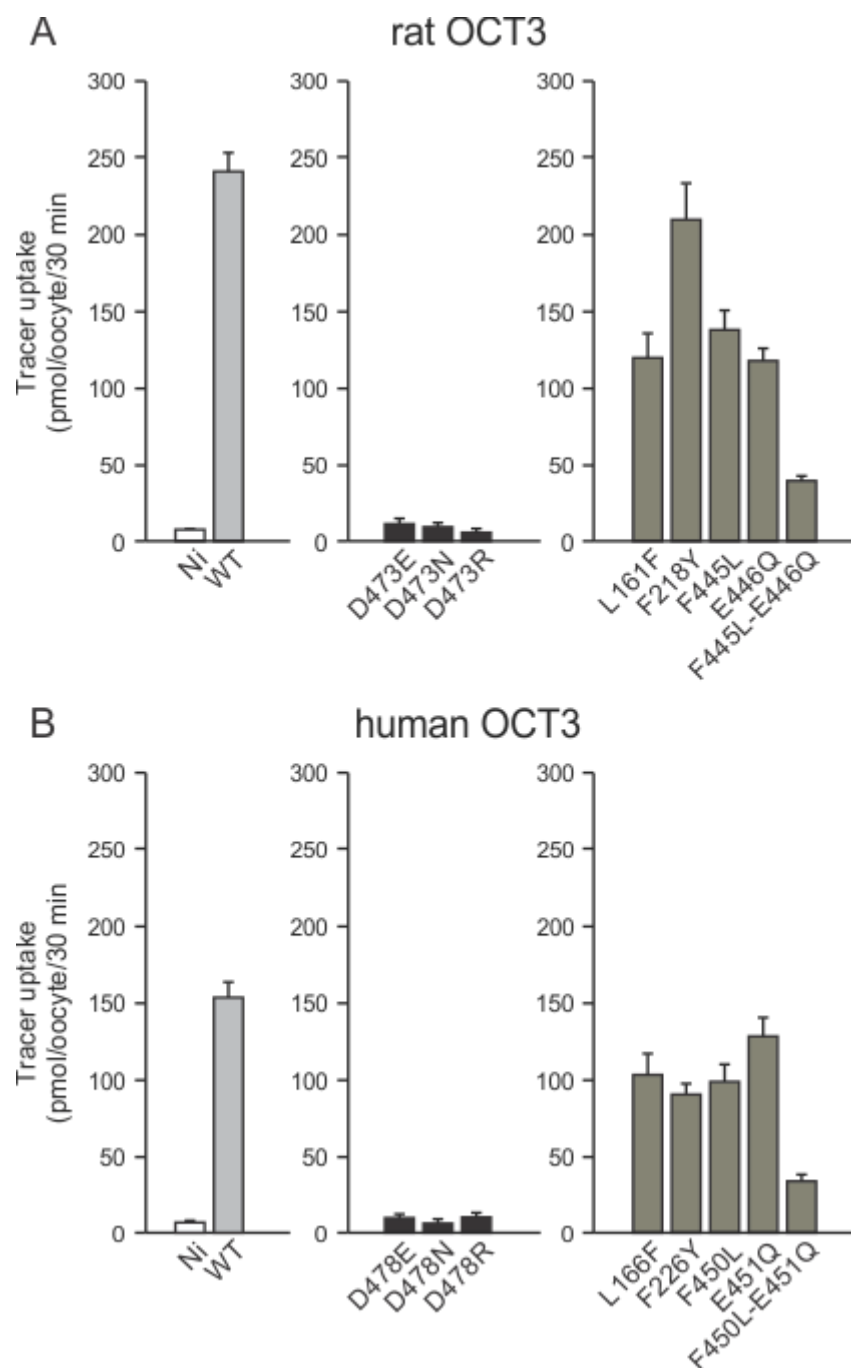
The uptake of 0.1  $\mu\text{M}$  [<sup>3</sup>H]MPP<sup>+</sup> into non-injected oocytes and into oocytes injected with rat and human isoforms of both wild-type and mutant OCT3 cRNA, was assessed (Figure 8). In non-injected oocytes, MPP<sup>+</sup> uptake was  $6.9 \pm 0.2$  pmol/oocyte/30 min.

In oocytes expressing wild-type rOCT3, MPP<sup>+</sup> transport rates were  $240.3 \pm 3.5$  pmol/oocyte/30 min, approximately 35-fold higher than in non-injected oocytes (Figure 8A, left panel). In contrast, mutations in D473 drastically reduced rOCT3-mediated uptake of MPP<sup>+</sup>: not only charge neutralization (D473N;  $9.8 \pm 2.0$ ) or charge reversal (D473R;  $8.7 \pm 2.5$ ), but also charge exchange (D473E;  $9.8 \pm 1.1$ ) (Figure 8A, middle panel). In oocytes expressing the wild-type hOCT3, MPP<sup>+</sup> transport rates were  $156.8 \pm 8.8$  pmol/oocyte/30 min, approximately 21-fold higher than in non-injection oocytes (Figure 8B, left panel). The same mutations were made for the corresponding aspartate residue in hOCT3 (D478). D478E, D478N, and D478R similarly abolished hOCT3-mediated transport, reducing MPP<sup>+</sup> uptake to  $8.7 \pm 1.3$ ,  $6.0 \pm 0.2$ , and  $7.9 \pm 0.8$  pmol/oocyte/30 min, respectively (Figure 8B, middle panel).

<b>Residue in rOCT1</b>	<b>Residue in OCT3 (rOCT3/hOCT3)</b>	<b>Mutations</b>	<b>Mutation Character</b>
<b>Hydrophobic Pocket Mutations (OCT3 → OCT1)</b>			
<b>F160</b>	L161/L166	Leu → Phe	Neutral → Aromatic
<b>F222</b>	Y218/Y226	Tyr → Phe	Aromatic → Aromatic
<b>L447</b>	F445/F4450	Phe → Leu	Aromatic → Neutral
<b>Q448</b>	E446/E451	Glu → Gln	Acidic → Polar
<b>Proposed Cation Binding Site Mutations</b>			
<b>D475</b>	D473/D478	Asp → Glu	Acidic → Acidic
		Asp → Gln	Acidic → Polar
		Asp → Arg	Acidic → Basic

**Table 3. Mutations in rOCT3 and hOCT3 Examined in this Study.**

Hydrophobic pocket mutations are at residues which have been identified by mutagenesis and rOCT1 homology modelling to be potential determinants of substrate affinity and/or sensitivity. Mutations in these residues in OCT3 are to the homologous residue in rOCT1. An aspartate residue is absolutely conserved in all OCT isoforms and has been proposed as the cationic binding site in rOCT1. Charge exchange, charge neutralization, and charge reversal mutations at this residue in OCT3 was examined.



**Figure 8. Transport of MPP<sup>+</sup> by rOCT3 and hOCT3 Mutants.**

Results are means  $\pm$  S.E. for 3 to 10 experiments from different oocyte preparations. Both **A.** and **B.** represent uptake of 0.1  $\mu$ M [<sup>3</sup>H]MPP<sup>+</sup> in non-injected oocytes (Ni, white bars), in oocytes expressing wild-type rOCT3 and hOCT3 (light grey bars), and in rOCT3 or hOCT3 mutants at the proposed cationic substrate binding residue (black bars) or residues lining the putative hydrophobic substrate binding pocket (dark grey bars).

These results suggest that this aspartate residue (D473 in rOCT and D478 in hOCT3) is an absolute requirement for OCT3-mediated transport and may participate in the stabilization of the positively-charged cationic component of MPP<sup>+</sup> in particular, and of OCT substrates in general. It has been observed previously that, in OCT1 and OCT2, D475 is critical for affinity and substrate selectivity (Gorboulev, Volk et al. 1999, Popp, Gorboulev et al. 2005, Higashi, Imamura et al. 2014). The observed reduction of MPP<sup>+</sup> uptake in the charge replacement mutation (Asp to Glu) implies that this aspartate residue not only may contribute to an electrostatic stabilization of positively-charged substrates, but also that the steric and spatial orientation of this interaction is critically important for the transport mechanism of both the rat and human isoforms of OCT3.

#### *MPP<sup>+</sup> Uptake by OCT3 Hydrophobic Binding Pocket Mutants*

Next, the uptake of 0.1  $\mu$ M [<sup>3</sup>H]MPP<sup>+</sup> into oocytes expressing mutations in rOCT3 and hOCT3 at residues lining the putative substrate binding pocket of the transporter was assessed (Figure 8A-8B). Mutation of these residues in rOCT3 and hOCT3 to their corresponding positions in rOCT1 (Table 3) decreased MPP<sup>+</sup> uptake rate (Figure 8A-8B, right panels). These results may reflect a shift in MPP<sup>+</sup> uptake rate toward that of OCT1 and OCT2, both of which have lower intrinsic MPP<sup>+</sup> uptake rates compared to OCT3, as was demonstrated in Part I (Figure 4A). Oocytes expressing mutant transporters displayed MPP<sup>+</sup> uptake rates at least five-fold above that of non-injected oocytes (Figure 8A-8B, right panels). Although the MPP<sup>+</sup> uptake rate decreased, thereby recapitulating the intrinsically lower transport rate of OCT1 and OCT2 (Figure 4A), the remaining OCT3-mediated activity in mutant transporters was sufficiently above the background as to allow for the same assessment of MPP<sup>+</sup> uptake inhibition as in Part I (Figure 5).

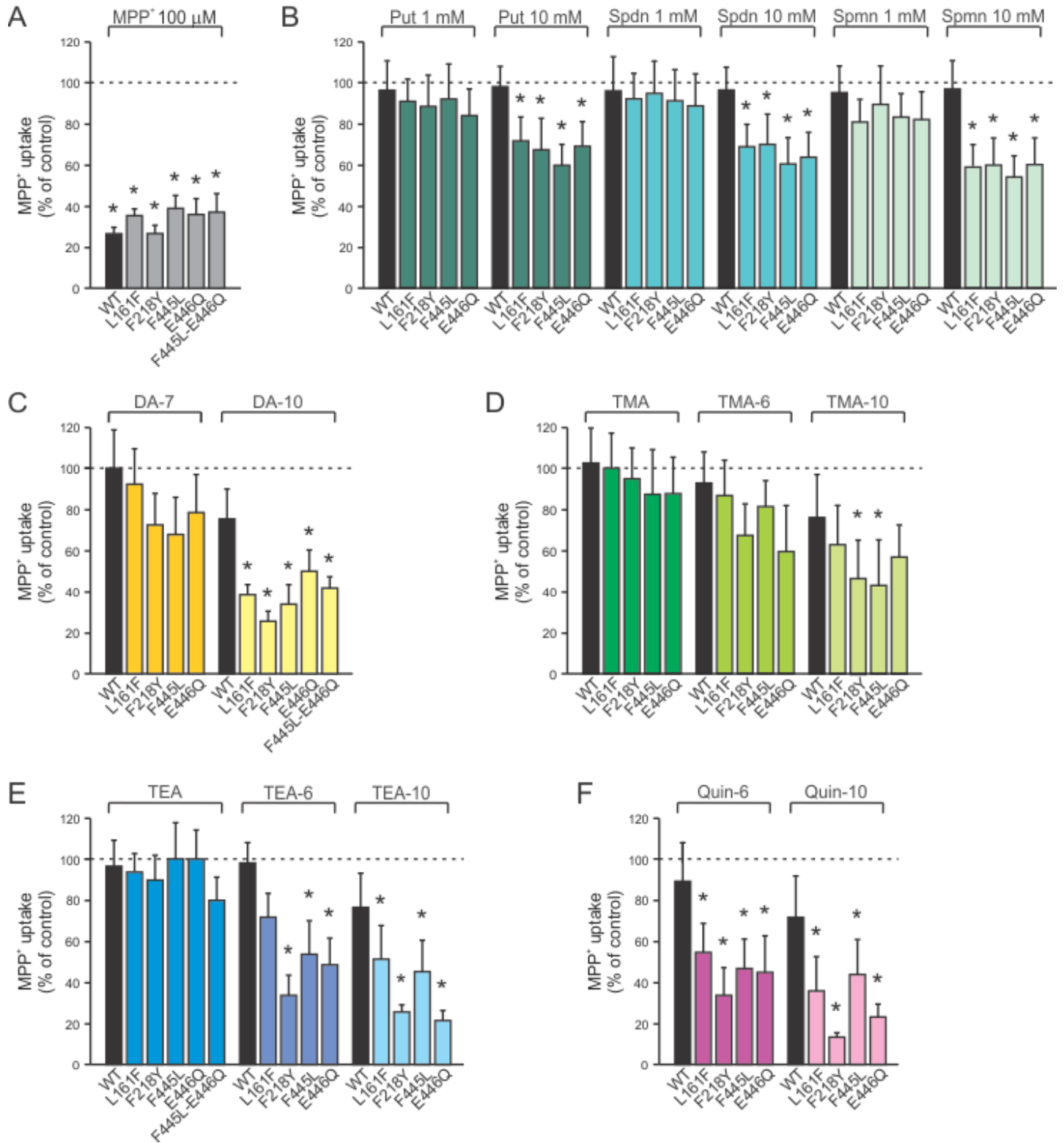
### *Inhibition of MPP<sup>+</sup> Uptake by Polyamines and Polyamine Analogs in OCT3 Mutants*

For further insight into changes in substrate specificity as a result of mutations of residues in the putative substrate binding pocket of OCT3, blocking potency of MPP<sup>+</sup> uptake was assessed for the same selection of structural analogs of the natural polyamines (putrescine, spermidine, and spermine) as in Part I (Figure 3). Each of these substrates was screened for effect on the uptake of 0.1  $\mu$ M [<sup>3</sup>H]MPP<sup>+</sup> into oocytes expressing wild-type and mutant rat and human OCT3 (Figure 9, Figure 10). Putrescine, spermidine, and spermine were added to the incubation buffer at 1 mM and 10 mM and unlabelled MPP<sup>+</sup> was added at its  $K_{0.5}$  value (0.1 mM). All other compounds were tested at 1 mM.

Uptake of [<sup>3</sup>H]MPP<sup>+</sup> in oocytes expressing wild-type and mutant rOCT3 or hOCT3 was inhibited about 50% by unlabeled MPP<sup>+</sup> (Figure 9A, Figure 10A). Consistent with the results of Part I (Figure 5), there was no measurable inhibition of MPP<sup>+</sup> uptake by natural polyamines (in particular, spermidine and spermine) for either rat or human wild-type OCT3. However, all mutations increased sensitivity to putrescine, spermidine and spermine in rOCT3 and hOCT3 with no discernible differences in blocking potency between any of the individual mutants or between polyamine species (Figure 9B, Figure 10B).

In Part I, it was observed that, for polyamine analogs, increasing hydrophobic character of the charged end groups and increasing degrees of acylation between them strengthens interaction with OCT1 and OCT2 (Figure 5A-5B), and that these requirements are accentuated in OCT3 (Figure 5C, Figure 9, Figure 10). In general, hydrophobic pocket-lining mutations resulted in increased sensitivity for the polyamine analogs, in particular for the decyl-compounds DA-10, TMA-10, TEA-10, and Quin-10, but also for the less hydrophobic hexyl-compounds TEA-6 and Quin-6 (Figure 9C-9F, Figure 10C-10F), which did not compete with MPP<sup>+</sup> in wild-type OCT3,

# rat OCT3

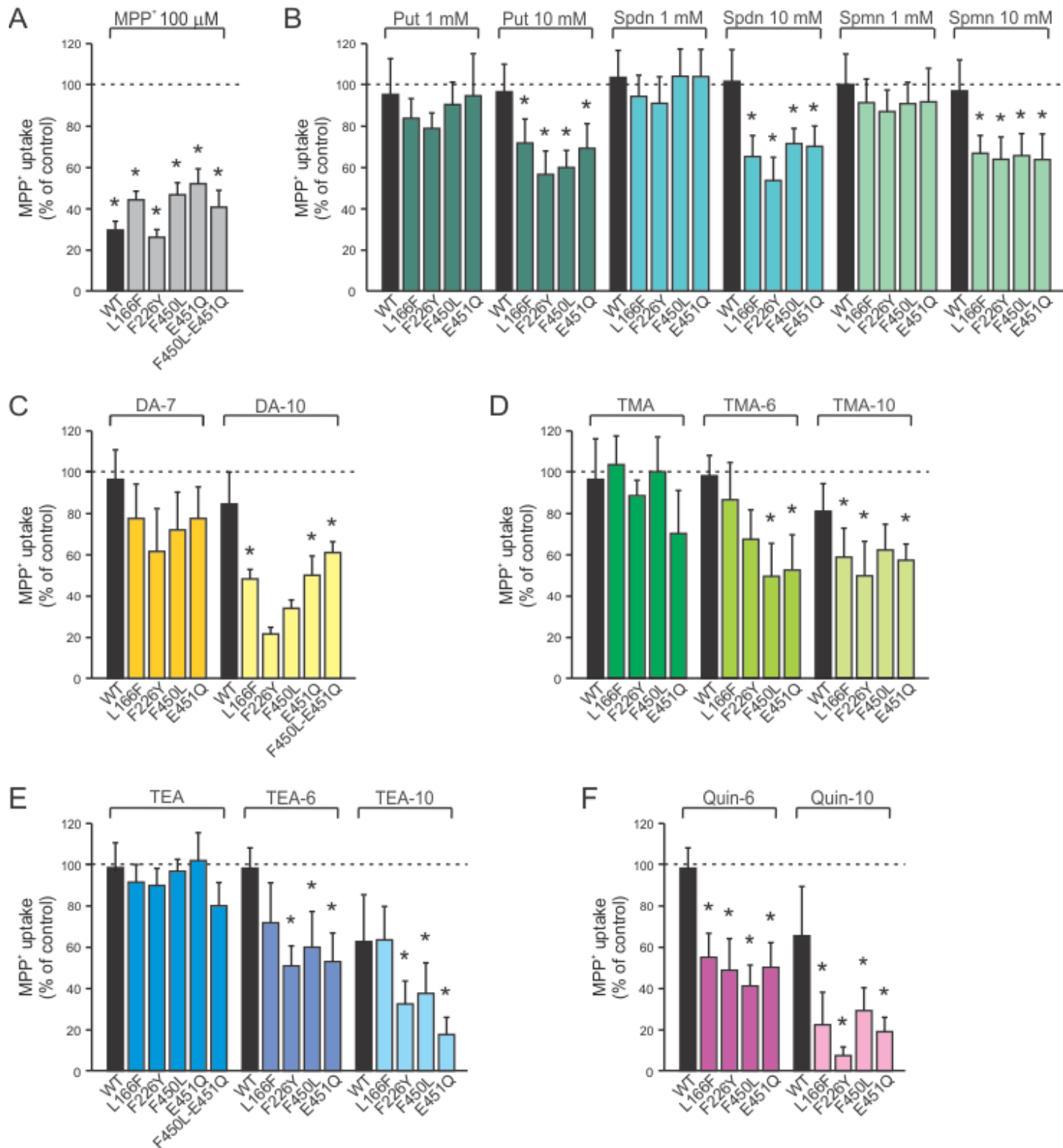




**Figure 9. Effect of Mutations in Rat OCT3 on Sensitivity to Model Cations, Polyamines, and Polyamine Analogs.**

Uptake of 0.1  $\mu\text{M}$  [ $^3\text{H}$ ]MPP $^+$  in oocytes expressing wild-type (black bars) and mutant rat OCT3 (L161F, F218Y, F445L, E446Q, and F445L-E446Q). Uptake was measured in the presence of **A.** MPP $^+$  (grey bars), **B.** the natural polyamines (putrescine, spermidine, and spermine; blue-green bars), **C.** diamine derivatives (DA-7 and DA-10; yellow bars), **D.** TMA and TMA derivatives (TMA-6 and TMA-10; green bars), **E.** TEA and TEA derivatives (TEA-6 and TEA-10; blue bars), or **F.** Quinuclidine derivatives (Quin-6 and Quin-10; purple bars). Uptake in non-injected oocytes was not affected by any of the test compounds. \* $p < 0.05$  as compared to maximal uptake in absence of external inhibitors (paired Student's  $t$  test).

# human OCT3



**Figure 10. Effect of Mutations in Human OCT3 on Sensitivity to Model Cations, Polyamines, and Polyamine Analogs.**

Uptake of 0.1  $\mu\text{M}$  [ $^3\text{H}$ ]MPP $^+$  in oocytes expressing wild-type (black bars) and mutant human OCT3 (L166F, F222Y, F450L, E451Q, and F450L-E451Q). Uptake was measured in the presence of **A.** MPP $^+$  (grey bars), **B.** the natural polyamines (putrescine, spermidine, and spermine; blue-green bars), **C.** diamine derivatives (DA-7 and DA-10; yellow bars), **D.** TMA and TMA derivatives (TMA-6 and TMA-10; green bars), **E.** TEA and TEA derivatives (TEA-6 and TEA-10; blue bars), or **F.** quinuclidine derivatives (Quin-6 and Quin-10; purple bars). Uptake in non-injected oocytes was not affected by any of the test compounds. \* $p < 0.05$  as compared to maximal uptake in absence of external inhibitors (paired Student's  $t$  test).

but did significantly block MPP<sup>+</sup> uptake in OCT1 and OCT2 (Figure 5). Finally, the isolated head group cations, TMA and TEA, did not have any effect on MPP<sup>+</sup> uptake in wild-type or mutant rOCT3 or hOCT3, implying that these mutations have not conferred any discernible change in the transporter's affinity and/or sensitivity for these small cationic compounds.

## DISCUSSION

### *A Conserved Aspartate is Essential for Transport by OCT3*

OCT1 and OCT2 have been biochemically well-characterized by previous mutagenesis studies which have identified an aspartate residue (D475 in rOCT1) on the predicted transmembrane (TM) helix 11 to be critically important for affinity and selectivity of substrates in OCT1 and OCT2 (Gorboulev, Volk et al. 1999, Popp, Gorboulev et al. 2005, Higashi, Imamura et al. 2014). The mechanism of substrate recognition and transport in OCT3, on the other hand, is comparatively more obscure as the set of biochemical data collected in systems expressing OCT3 is, at present, extremely limited. In this part, the hypothesis was tested that the aspartate residue identified in rOCT1 as critical to substrate transport, and which is absolutely conserved across OCT isoforms (Figure 7), performs a similar function in OCT3.

Here, it is shown that this conserved aspartate residue is indeed essential for transport in OCT3. Thus, mutation of this residue in rOCT3 (D473) and in hOCT3 (D478) resulted in complete abolition of carrier-mediated activity (Figure 8, middle panels). This drastic reduction in MPP<sup>+</sup> uptake is in support of the hypothesis that this TM11 Asp functions in all OCT isoforms as the singular binding site for the cationic region of transported substrates (Koepsell 2011). Although some models of substrate binding have been generated, in particular of spermidine binding to hOCT2 (Higashi, Imamura et al. 2014), questions regarding the exact role of this conserved residue

in substrate recognition persist. In particular, the present study suggests that simply the presence of a negative charge in that position is not sufficient to accomplish substrate binding and translocation, since the substitution of Glu for Asp results in a complete loss of function (Figure 8, middle panels). Rather, the unique spatial arrangements provided by the specific side chain of Asp in this position may be required for substrate coordination and further stabilization of the substrate's positive charge.

#### *Hydrophobic Pocket-Lining Residues Account for Differences in Substrate Selectivity between OCT1 and OCT3*

Homology modeling of rOCT1 has suggested the existence of a large hydrophobic cleft within which certain amino acids underlie substrate-transporter interactions (Figure 6) (Popp, Gorboulev et al. 2005, Volk, Gorboulev et al. 2009). However, key residues within this pocket are not conserved in other isoforms, in particular OCT3 (Figure 7). The present study addresses two outstanding questions: first, what the effect of mutagenesis at these residues is on the transport affinity or selectivity in OCT3; and second, whether changes at these residues may contribute to the observed differences in substrate selectivity (Sala-Rabanal, Li et al. 2013).

In general, replacing putative pocket-lining residues in rat or human OCT3 with their OCT1 counterparts – for example, L166F, F226Y, F450L, and E451Q in hOCT3 – had the effect of increasing the sensitivity of OCT3s to polyamines and polyamine analogs, which interacted poorly with the wild-type but were able to block MPP<sup>+</sup> uptake in mutant transporters (Figure 9, Figure 10), as they did in OCT1 and OCT2 (Figure 5).

For the natural polyamines – spermidine and spermine, in particular – it was observed in Part I that they did not inhibit MPP<sup>+</sup> transport in either OCT1, OCT2, or OCT3 at 1 mM (Figure

5). However it was also observed that at 10 mM, MPP<sup>+</sup> uptake was significantly blocked by OCT1 and OCT2, but not OCT3 (Figure 5). In this part, it is shown that mutations at any of the four putative binding pocket residues in rOCT3 or hOCT3 to their corresponding residues in rOCT1 result in inhibition of MPP<sup>+</sup> uptake by putrescine, spermidine, and spermine at 10 mM (Figure 9B, Figure 10B). This gain of polyamine sensitivity in the putative binding pocket mutants at 10 mM thus at least partially recapitulates the substrate specificity profile of OCT1 and OCT2 with respect to natural polyamine sensitivity.

Additionally, nearly all binding pocket mutants in OCT3 showed significant inhibition of MPP<sup>+</sup> uptake in the presence of the decyl-polyamine analog compounds (DA-10, TMA-10, TEA-10, and Quin-10) (Figure 9C-9F, Figure 10C-10F) with block by decyl-polyamine analogs being observed to be as high as 90% for the decyl-quinuclidine compounds in both rOCT3 and hOCT3 (Figure 9F, Figure 10F). Although wild-type OCT3 was observed to have about 50-60% block of MPP<sup>+</sup> uptake by the highly hydrophobic Quin-10, the degree of that block was significantly weaker than that of OCT1 and OCT2, whose MPP<sup>+</sup> uptake was 90% blocked by Quin-10 (Figure 5). In mutant OCT3, however, inhibition of MPP<sup>+</sup> uptake by Quin-10 in both rat and human isoforms was in the 80-90% range (Figure 9F, Figure 10F), similar to the range of MPP<sup>+</sup> block values observed in OCT1 and OCT2 by Quin-10 (Figure 5). A similar trend is also observed in the decyl TEA compound, TEA-10 (Figure 9E, Figure 10E), indicating a robust phenotypic shift toward the substrate sensitivity profiles of OCT1 and OCT2 in mutant rOCT3 and hOCT3.

Although a good deal of overlap exists between the range of blocking potencies of shorter chain polyamine analogs (DA-7, TMA-6, TEA-6, and Quin-10) and their decyl counterparts (DA-10, TMA-10, TEA-10, and Quin-10), a general trend of increasing blocking potency can be observed when moving from the shorter chain compounds to more acylated analogs within groups

(Figure 9C-9F, Figure 10C-10F). Notably, this trend is also observed in the substrate selectivity profiles of OCT1 and OCT2 where significant inhibition of MPP<sup>+</sup> uptake can be observed in the shorter chain polyamine analogs (DA-7, TMA-6, TEA-6, and Quin-6), but for which there is generally little observable MPP<sup>+</sup> block in wild-type OCT3 (Figure 5). In the putative binding pocket mutants of rat and human OCT3, significant inhibition of MPP<sup>+</sup> uptake is consistently observed in the short-chained TEA-6 and Quin-6, and is observed for most of the mutations in TMA-6 (Figure 9D-9F, Figure 10D-10F).

It is important to note that each of these single residue mutations in rat or human OCT3 represent one of many residues which purportedly contribute to determining substrate selectivity in OCTs (Gorboulev, Volk et al. 1999, Bednarczyk, Ekins et al. 2003, Popp, Gorboulev et al. 2005, Ahlin, Karlsson et al. 2008, Gorbunov, Gorboulev et al. 2008, Volk, Gorboulev et al. 2009, Koepsell 2011, Higashi, Imamura et al. 2014). Therefore, it is unlikely that any one mutant can completely reproduce the substrate selectivity profile of OCT1 or OCT2. However, in this part, it has been demonstrated that mutations at putative hydrophobic cleft-lining residues (L161F, F222Y, F445L, and E446Q in rOCT3 and L166F, F226Y, F450L, and E451Q in hOCT3) shift the substrate selectivity profile of rat and human OCT3 toward the higher sensitivity phenotypes observed in OCT1 and OCT2. The results of the present study have therefore shown that the set of putative binding pocket-lining residues examined here are at least in part responsible for the observed differences in substrate selectivity between OCT isoforms, which may also provide important insights into the mechanism and structural determinants of substrate recognition and translocation in OCTs in general.

## REFERENCES

- Abramson, J., I. Smirnova, V. Kasho, G. Verner, H. R. Kaback and S. Iwata (2003). "Structure and mechanism of the lactose permease of *Escherichia coli*." Science **301**(5633): 610-615.
- Ahlin, G., J. Karlsson, J. M. Pedersen, L. Gustavsson, R. Larsson, P. Matsson, U. Norinder, C. A. Bergstrom and P. Artursson (2008). "Structural requirements for drug inhibition of the liver specific human organic cation transport protein 1." J Med Chem **51**(19): 5932-5942.
- Aouida, M., A. Leduc, R. Poulin and D. Ramotar (2005). "AGP2 encodes the major permease for high affinity polyamine import in *Saccharomyces cerevisiae*." J Biol Chem **280**(25): 24267-24276.
- Bednarczyk, D., S. Ekins, J. H. Wikel and S. H. Wright (2003). "Influence of molecular structure on substrate binding to the human organic cation transporter, hOCT1." Mol Pharmacol **63**(3): 489-498.
- Benedikt, J., M. Inyushin, Y. V. Kucheryavykh, Y. Rivera, L. Y. Kucheryavykh, C. G. Nichols, M. J. Eaton and S. N. Skatchkov (2012). "Intracellular polyamines enhance astrocytic coupling." Neuroreport **23**(17): 1021-1025.
- Bockenbauer, D., S. Feather, H. C. Stanescu, S. Bandulik, A. A. Zdebik, M. Reichold, J. Tobin, E. Lieberer, C. Sterner, G. Landouere, R. Arora, T. Sirimanna, D. Thompson, J. H. Cross, W. van't Hoff, O. Al Masri, K. Tullus, S. Yeung, Y. Anikster, E. Klootwijk, M. Hubank, M. J. Dillon, D. Heitzmann, M. Arcos-Burgos, M. A. Knepper, A. Dobbie, W. A. Gahl, R. Warth, E. Sheridan and R. Kleta (2009). "Epilepsy, ataxia, sensorineural deafness, tubulopathy, and KCNJ10 mutations." N Engl J Med **360**(19): 1960-1970.



- Brast, S., A. Grabner, S. Sucic, H. H. Sitte, E. Hermann, H. Pavenstadt, E. Schlatter and G. Ciarimboli (2012). "The cysteines of the extracellular loop are crucial for trafficking of human organic cation transporter 2 to the plasma membrane and are involved in oligomerization." FASEB J **26**(3): 976-986.
- Budiman, T., E. Bamberg, H. Koepsell and G. Nagel (2000). "Mechanism of electrogenic cation transport by the cloned organic cation transporter 2 from rat." J Biol Chem **275**(38): 29413-29420.
- Burckhardt, B. C. and P. Thelen (1995). "Effect of primary, secondary and tertiary amines on membrane potential and intracellular pH in *Xenopus laevis* oocytes." Pflugers Arch **429**(3): 306-312.
- Busch, A. E., U. Karbach, D. Miska, V. Gorboulev, A. Akhoundova, C. Volk, P. Arndt, J. C. Ulzheimer, M. S. Sonders, C. Baumann, S. Waldegger, F. Lang and H. Koepsell (1998). "Human neurons express the polyspecific cation transporter hOCT2, which translocates monoamine neurotransmitters, amantadine, and memantine." Mol Pharmacol **54**(2): 342-352.
- Busch, A. E., S. Quester, J. C. Ulzheimer, V. Gorboulev, A. Akhoundova, S. Waldegger, F. Lang and H. Koepsell (1996). "Monoamine neurotransmitter transport mediated by the polyspecific cation transporter rOCT1." FEBS Lett **395**(2-3): 153-156.
- Busch, A. E., S. Quester, J. C. Ulzheimer, S. Waldegger, V. Gorboulev, P. Arndt, F. Lang and H. Koepsell (1996). "Electrogenic properties and substrate specificity of the polyspecific rat cation transporter rOCT1." J Biol Chem **271**(51): 32599-32604.
- Casero, R. A., Jr. and L. J. Marton (2007). "Targeting polyamine metabolism and function in cancer and other hyperproliferative diseases." Nat Rev Drug Discov **6**(5): 373-390.

- Casero, R. A. and A. E. Pegg (2009). "Polyamine catabolism and disease." Biochem J **421**(3): 323-338.
- Chen, Y., S. Zhang, M. Sorani and K. M. Giacomini (2007). "Transport of paraquat by human organic cation transporters and multidrug and toxic compound extrusion family." J Pharmacol Exp Ther **322**(2): 695-700.
- Choi, M. K. and I. S. Song (2008). "Organic cation transporters and their pharmacokinetic and pharmacodynamic consequences." Drug Metab Pharmacokinet **23**(4): 243-253.
- Ciarimboli, G. (2011). "Role of organic cation transporters in drug-induced toxicity." Expert Opin Drug Metab Toxicol **7**(2): 159-174.
- Cui, M., R. Aras, W. V. Christian, P. M. Rappold, M. Hatwar, J. Panza, V. Jackson-Lewis, J. A. Javitch, N. Ballatori, S. Przedborski and K. Tieu (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**(19): 8043-8048.
- Dresser, M. J., A. T. Gray and K. M. Giacomini (2000). "Kinetic and selectivity differences between rodent, rabbit, and human organic cation transporters (OCT1)." J Pharmacol Exp Ther **292**(3): 1146-1152.
- Enkvetchakul, D., L. Ebihara and C. G. Nichols (2003). "Polyamine flux in *Xenopus* oocytes through hemi-gap junctional channels." J Physiol **553**(Pt 1): 95-100.
- Gorboulev, V., N. Shatskaya, C. Volk and H. Koepsell (2005). "Subtype-specific affinity for corticosterone of rat organic cation transporters rOCT1 and rOCT2 depends on three amino acids within the substrate binding region." Mol Pharmacol **67**(5): 1612-1619.

- Gorboulev, V., J. C. Ulzheimer, A. Akhoundova, I. Ulzheimer-Teuber, U. Karbach, S. Quester, C. Baumann, F. Lang, A. E. Busch and H. Koepsell (1997). "Cloning and characterization of two human polyspecific organic cation transporters." DNA Cell Biol **16**(7): 871-881.
- Gorboulev, V., C. Volk, P. Arndt, A. Akhoundova and H. Koepsell (1999). "Selectivity of the polyspecific cation transporter rOCT1 is changed by mutation of aspartate 475 to glutamate." Mol Pharmacol **56**(6): 1254-1261.
- Gorbunov, D., V. Gorboulev, N. Shatskaya, T. Mueller, E. Bamberg, T. Friedrich and H. Koepsell (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**(1): 50-61.
- Grundemann, D., C. Hahne, R. Berkels and E. Schomig (2003). "Agmatine is efficiently transported by non-neuronal monoamine transporters extraneuronal monoamine transporter (EMT) and organic cation transporter 2 (OCT2)." J Pharmacol Exp Ther **304**(2): 810-817.
- Harlfinger, S., C. Fork, A. Lazar, E. Schomig and D. Grundemann (2005). "Are organic cation transporters capable of transporting prostaglandins?" Naunyn Schmiedebergs Arch Pharmacol **372**(2): 125-130.
- Hendrickx, R., J. G. Johansson, C. Lohmann, R. M. Jenvert, A. Blomgren, L. Borjesson and L. Gustavsson (2013). "Identification of novel substrates and structure-activity relationship of cellular uptake mediated by human organic cation transporters 1 and 2." J Med Chem **56**(18): 7232-7242.

- Hibino, H., A. Inanobe, K. Furutani, S. Murakami, I. Findlay and Y. Kurachi (2010). "Inwardly rectifying potassium channels: their structure, function, and physiological roles." Physiol Rev **90**(1): 291-366.
- Higashi, K., M. Imamura, S. Fudo, T. Uemura, R. Saiki, T. Hoshino, T. Toida, K. Kashiwagi and K. Igarashi (2014). "Identification of functional amino acid residues involved in polyamine and agmatine transport by human organic cation transporter 2." PLoS One **9**(7): e102234.
- Hosoya, K. and M. Tachikawa (2011). "Roles of organic anion/cation transporters at the blood-brain and blood-cerebrospinal fluid barriers involving uremic toxins." Clin Exp Nephrol **15**(4): 478-485.
- Huang, Y., M. J. Lemieux, J. Song, M. Auer and D. N. Wang (2003). "Structure and mechanism of the glycerol-3-phosphate transporter from Escherichia coli." Science **301**(5633): 616-620.
- Igarashi, K. and K. Kashiwagi (2010). "Modulation of cellular function by polyamines." Int J Biochem Cell Biol **42**(1): 39-51.
- Inazu, M., H. Takeda and T. Matsumiya (2003). "Expression and functional characterization of the extraneuronal monoamine transporter in normal human astrocytes." J Neurochem **84**(1): 43-52.
- Inyushin, M., L. Y. Kucheryavykh, Y. V. Kucheryavykh, C. G. Nichols, R. J. Buono, T. N. Ferraro, S. N. Skatchkov and M. J. Eaton (2010). "Potassium channel activity and glutamate uptake are impaired in astrocytes of seizure-susceptible DBA/2 mice." Epilepsia **51**(9): 1707-1713.

- Isome, M., M. J. Lortie, Y. Murakami, E. Parisi, S. Matsufuji and J. Satriano (2007). "The antiproliferative effects of agmatine correlate with the rate of cellular proliferation." Am J Physiol Cell Physiol **293**(2): C705-711.
- Keller, T., B. Egenberger, V. Gorboulev, F. Bernhard, Z. Uzelac, D. Gorbunov, C. Wirth, S. Koppatz, V. Dotsch, C. Hunte, H. H. Sitte and H. Koepsell (2011). "The large extracellular loop of organic cation transporter 1 influences substrate affinity and is pivotal for oligomerization." J Biol Chem **286**(43): 37874-37886.
- Keller, T., M. Elfeber, V. Gorboulev, H. Reilander and H. Koepsell (2005). "Purification and functional reconstitution of the rat organic cation transporter OCT1." Biochemistry **44**(36): 12253-12263.
- Koepsell, H. (2004). "Polyspecific organic cation transporters: their functions and interactions with drugs." Trends Pharmacol Sci **25**(7): 375-381.
- Koepsell, H. (2011). "Substrate recognition and translocation by polyspecific organic cation transporters." Biol Chem **392**(1-2): 95-101.
- Koepsell, H., K. Lips and C. Volk (2007). "Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications." Pharm Res **24**(7): 1227-1251.
- Kucheryavykh, Y. V., Y. M. Shuba, S. M. Antonov, M. Y. Inyushin, L. Cubano, W. L. Pearson, H. Kurata, A. Reichenbach, R. W. Veh, C. G. Nichols, M. J. Eaton and S. N. Skatchkov (2008). "Complex rectification of Muller cell Kir currents." Glia **56**(7): 775-790.

- Kurata, H. T., L. R. Phillips, T. Rose, G. Loussouarn, S. Herlitze, H. Fritzenschaft, D. Enkvetchakul, C. G. Nichols and T. Baukowitz (2004). "Molecular basis of inward rectification: polyamine interaction sites located by combined channel and ligand mutagenesis." J Gen Physiol **124**(5): 541-554.
- Li, D. C., C. G. Nichols and M. Sala-Rabanal (2015). "Molecular Determinants of Substrate Selectivity in OCT3 (SLC22A3)." Biophys J **108**(2): 461a.
- Lin, C. J., Y. Tai, M. T. Huang, Y. F. Tsai, H. J. Hsu, K. Y. Tzen and H. H. Liou (2010). "Cellular localization of the organic cation transporters, OCT1 and OCT2, in brain microvessel endothelial cells and its implication for MPTP transport across the blood-brain barrier and MPTP-induced dopaminergic toxicity in rodents." J Neurochem **114**(3): 717-727.
- Lips, K. S., C. Volk, B. M. Schmitt, U. Pfeil, P. Arndt, D. Miska, L. Ermert, W. Kummer and H. Koepsell (2005). "Polyspecific cation transporters mediate luminal release of acetylcholine from bronchial epithelium." Am J Respir Cell Mol Biol **33**(1): 79-88.
- Loussouarn, G., L. J. Marton and C. G. Nichols (2005). "Molecular basis of inward rectification: structural features of the blocker defined by extended polyamine analogs." Mol Pharmacol **68**(2): 298-304.
- Ming, X., W. Ju, H. Wu, R. R. Tidwell, J. E. Hall and D. R. Thakker (2009). "Transport of dicationic drugs pentamidine and furamidine by human organic cation transporters." Drug Metab Dispos **37**(2): 424-430.
- Muller, J., K. S. Lips, L. Metzner, R. H. Neubert, H. Koepsell and M. Brandsch (2005). "Drug specificity and intestinal membrane localization of human organic cation transporters (OCT)." Biochem Pharmacol **70**(12): 1851-1860.

- Nagel, G., C. Volk, T. Friedrich, J. C. Ulzheimer, E. Bamberg and H. Koepsell (1997). "A reevaluation of substrate specificity of the rat cation transporter rOCT1." J Biol Chem **272**(51): 31953-31956.
- Nichols, C. G. and A. N. Lopatin (1997). "Inward rectifier potassium channels." Annu Rev Physiol **59**: 171-191.
- Nies, A. T., H. Koepsell, K. Damme and M. Schwab (2011). "Organic cation transporters (OCTs, MATEs), in vitro and in vivo evidence for the importance in drug therapy." Handb Exp Pharmacol(201): 105-167.
- Nishimura, M. and S. Naito (2005). "Tissue-specific mRNA expression profiles of human ATP-binding cassette and solute carrier transporter superfamilies." Drug Metab Pharmacokinet **20**(6): 452-477.
- Okuda, M., Y. Urakami, H. Saito and K. Inui (1999). "Molecular mechanisms of organic cation transport in OCT2-expressing *Xenopus* oocytes." Biochim Biophys Acta **1417**(2): 224-231.
- Pegg, A. E. (2009). "Mammalian polyamine metabolism and function." IUBMB Life **61**(9): 880-894.
- Popp, C., V. Gorboulev, T. D. Muller, D. Gorbunov, N. Shatskaya and H. Koepsell (2005). "Amino acids critical for substrate affinity of rat organic cation transporter 1 line the substrate binding region in a model derived from the tertiary structure of lactose permease." Mol Pharmacol **67**(5): 1600-1611.
- Poulin, R., R. A. Casero and D. Soulet (2012). "Recent advances in the molecular biology of metazoan polyamine transport." Amino Acids **42**(2-3): 711-723.

- Rock, D. M. and R. L. Macdonald (1995). "Polyamine regulation of N-methyl-D-aspartate receptor channels." Annu Rev Pharmacol Toxicol **35**: 463-482.
- Sala-Rabanal, M., L. Y. Kucheryavykh, S. N. Skatchkov, M. J. Eaton and C. G. Nichols (2010). "Molecular mechanisms of EAST/SeSAME syndrome mutations in Kir4.1 (KCNJ10)." J Biol Chem **285**(46): 36040-36048.
- Sala-Rabanal, M., D. C. Li, G. R. Dake, H. T. Kurata, M. Inyushin, S. N. Skatchkov and C. G. Nichols (2013). "Polyamine transport by the polyspecific organic cation transporters OCT1, OCT2, and OCT3." Mol Pharm **10**(4): 1450-1458.
- Sata, R., H. Ohtani, M. Tsujimoto, H. Murakami, N. Koyabu, T. Nakamura, T. Uchiumi, M. Kuwano, H. Nagata, K. Tsukimori, H. Nakano and Y. Sawada (2005). "Functional analysis of organic cation transporter 3 expressed in human placenta." J Pharmacol Exp Ther **315**(2): 888-895.
- Schmitt, B. M. and H. Koepsell (2005). "Alkali cation binding and permeation in the rat organic cation transporter rOCT2." J Biol Chem **280**(26): 24481-24490.
- Scholl, U. I., M. Choi, T. Liu, V. T. Ramaekers, M. G. Hausler, J. Grimmer, S. W. Tobe, A. Farhi, C. Nelson-Williams and R. P. Lifton (2009). "Seizures, sensorineural deafness, ataxia, mental retardation, and electrolyte imbalance (SeSAME syndrome) caused by mutations in KCNJ10." Proc Natl Acad Sci U S A **106**(14): 5842-5847.
- Sha, Q., C. Romano, A. N. Lopatin and C. G. Nichols (1996). "Spermidine release from xenopus oocytes. Electrodifussion through a membrane channel." J Biol Chem **271**(7): 3392-3397.
- Sobczak, K., N. Bangel-Ruland, G. Leier and W. M. Weber (2010). "Endogenous transport systems in the *Xenopus laevis* oocyte plasma membrane." Methods **51**(1): 183-189.



- Suhre, W. M., S. Ekins, C. Chang, P. W. Swaan and S. H. Wright (2005). "Molecular determinants of substrate/inhibitor binding to the human and rabbit renal organic cation transporters hOCT2 and rbOCT2." Mol Pharmacol **67**(4): 1067-1077.
- Uemura, T., H. F. Yerushalmi, G. Tsaprailis, D. E. Stringer, K. E. Pastorian, L. Hawel, 3rd, C. V. Byus and E. W. Gerner (2008). "Identification and characterization of a diamine exporter in colon epithelial cells." J Biol Chem **283**(39): 26428-26435.
- Van Dobbenburgh, O. A., B. Houwen, H. Jurjens, J. Marrink, M. R. Halie and H. O. Nieweg (1983). "Plasma spermidine concentrations as early indication of response to therapy in human myeloma." J Clin Pathol **36**(7): 804-807.
- Volk, C., V. Gorboulev, A. Kotzsch, T. D. Muller and H. Koepsell (2009). "Five amino acids in the innermost cavity of the substrate binding cleft of organic cation transporter 1 interact with extracellular and intracellular corticosterone." Mol Pharmacol **76**(2): 275-289.
- Wang, J. F., R. B. Su, N. Wu, B. Xu, X. Q. Lu, Y. Liu and J. Li (2005). "Inhibitory effect of agmatine on proliferation of tumor cells by modulation of polyamine metabolism." Acta Pharmacol Sin **26**(5): 616-622.
- Weiger, T. M., T. Langer and A. Hermann (1998). "External action of di- and polyamines on maxi calcium-activated potassium channels: an electrophysiological and molecular modeling study." Biophys J **74**(2 Pt 1): 722-730.
- Winter, T. N., W. F. Elmquist and C. A. Fairbanks (2011). "OCT2 and MATE1 provide bidirectional agmatine transport." Mol Pharm **8**(1): 133-142.
- Wright, S. H. (2005). "Role of organic cation transporters in the renal handling of therapeutic agents and xenobiotics." Toxicol Appl Pharmacol **204**(3): 309-319.

- Wright, S. H. and W. H. Dantzer (2004). "Molecular and cellular physiology of renal organic cation and anion transport." Physiol Rev **84**(3): 987-1049.
- Zhang, L., W. Gorset, M. J. Dresser and K. M. Giacomini (1999). "The interaction of n-tetraalkylammonium compounds with a human organic cation transporter, hOCT1." J Pharmacol Exp Ther **288**(3): 1192-1198.
- Zhang, X., N. V. Shirahatti, D. Mahadevan and S. H. Wright (2005). "A conserved glutamate residue in transmembrane helix 10 influences substrate specificity of rabbit OCT2 (SLC22A2)." J Biol Chem **280**(41): 34813-34822.
- Zolk, O. (2012). "Disposition of metformin: variability due to polymorphisms of organic cation transporters." Ann Med **44**(2): 119-129.
- Zolk, O., T. F. Solbach, J. König and M. F. Fromm (2009). "Structural determinants of inhibitor interaction with the human organic cation transporter OCT2 (SLC22A2)." Naunyn Schmiedebergs Arch Pharmacol **379**(4): 337-348.