

# Activation of the extraneuronal monoamine transporter (EMT) from rat expressed in 293 cells

\*<sup>1</sup>Dirk Gründemann, <sup>1</sup>Ann-Cathrin Koschker, <sup>1</sup>Christine Haag, <sup>1</sup>Cornelius Honold, <sup>1</sup>Tim Zimmermann & <sup>1</sup>Edgar Schömig

<sup>1</sup>Department of Pharmacology, University of Cologne, Gleueler Strasse 24, 50931 Cologne, Germany

**1** The extraneuronal monoamine transporter from rat (EMTr) was heterologously expressed by stable transfection in human embryonic kidney 293 cells and characterized in radiotracer experiments.

**2** EMTr-mediated uptake of 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) was saturable, with a  $K_m$  of 151  $\mu\text{mol l}^{-1}$  and  $V_{\text{max}}$  of 7.5  $\text{nmol min}^{-1} \text{mg protein}^{-1}$ .

**3** Compared to the human orthologue EMTh (gene symbol *SLC22A3*), EMTr was about two orders of magnitude more resistant to most inhibitors, including disprocynium24 and corticosterone.

**4** Strikingly, inhibitors and substrates at low concentration stimulated EMTr-mediated transport above control level with MPP<sup>+</sup> and noradrenaline as substrate, but not with cimetidine. Results were confirmed with EMT from mouse.

**5** With different  $\text{IC}_{50}$ -values for different substrates, the standard method to calculate  $K_i$ -values is not applicable.

**6** Our experiments suggest that activation is not caused by changes in membrane potential or *trans*-stimulation. Since the extent of activation depends markedly on the chemical structure of the monitored substrate, involvement of a receptor-mediated signalling pathway or recruitment of transporter reserve are implausible.

**7** To explain activation, we present a kinetic model which assumes two binding sites for substrate or inhibitor per transporter entity, possibly resulting from the assembly of homodimers.

**8** Activation explains previous reports about inhibitor-insensitive catecholamine transport in rat brain.

**9** We speculate that activation may serve to keep the transporter working for specific substrates in the face of inhibitors.

*British Journal of Pharmacology* (2002) 137, 910–918. doi:10.1038/sj.bjp.0704926

**Keywords:** Extraneuronal monoamine transporter; transporter activation; 1-methyl-4-phenylpyridinium; organic cation transporter; catecholamine transport; uptake<sub>2</sub>; noradrenaline; cimetidine; *trans*-stimulation;  $K_i$ -value

**Abbreviations:** EMT, extraneuronal monoamine transporter; kb, kilobase(s); MPP<sup>+</sup>, 1-methyl-4-phenylpyridinium; OCT, organic cation transporter; PCR, polymerase chain reaction; RT, reverse transcriptase; h, r, and m attached to a protein name designate species as being human, rat, or mouse, respectively

## Introduction

At the plasma membrane of neuronal and non-neuronal cells, specific transport proteins remove released monoamine transmitters, such as dopamine, noradrenaline, and 5-hydroxytryptamine, from the extracellular space and thus terminate and control signal transmission. Pharmacological blockade of these carriers serves to increase extracellular transmitter levels. The therapeutic utility of this principle is well acknowledged for neuronal transporters, which are molecular targets for e.g. clinically important antidepressants. We have a long-standing interest in the counterpart from non-neuronal cells, the extraneuronal monoamine transporter (EMT), originally discovered as a transport mechanism in rat heart and designated 'uptake<sub>2</sub>' by Iversen and coworkers (Iversen, 1965). As part of both the central and peripheral aminergic nervous system (Eisenhofer *et al.*, 1996; Russ *et al.*,

1996), EMT could be involved in a number of clinically prominent human diseases, e.g. Parkinson's disease, schizophrenia, hypertension, and arrhythmia.

The functional characterization of EMT was pioneered with perfused organs and isolated tissues by the groups of Iversen and Trendelenburg (Grohmann & Trendelenburg, 1984; Iversen & Salt, 1970; Trendelenburg, 1988). Functional studies with neurotoxin 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) as excellent substrate on Caki-1 cells, a human kidney carcinoma cell line, have complemented the picture (Russ *et al.*, 1992; Schömig *et al.*, 1992; Schömig & Schönfeld, 1990). Based on this cell culture model, our group has developed highly potent and specific inhibitors for EMT, e.g., disprocynium24 and decynium22 (Russ *et al.*, 1993). It is clear now that EMT differs immensely with respect to transport mechanism, drug sensitivity, substrate affinity, and substrate selectivity from the neuronal transporters e.g. for dopamine (DAT) and noradrenaline (NET) (Povlock & Amara, 1997).

\*Author for correspondence;  
E-mail: dirk.gruendemann@uni-koeln.de

Recently, we have succeeded in the molecular identification of human EMT (gene symbol *SLC22A3*) by cloning from a Caki-1 cDNA library and functional expression in 293 cells (Gründemann *et al.*, 1998b). This assignment was thereafter confirmed for orthologous cDNAs from rat (Kekuda *et al.*, 1998; Wu *et al.*, 1998) and mouse (Verhaagh *et al.*, 1999; Wu *et al.*, 2000). It has become evident by molecular cloning and by functional characterization based on heterologous expression that transporters in addition to EMT may participate in non-neuronal monoamine uptake, i.e. OCT1 (Breider *et al.*, 1998; Gründemann *et al.*, 1994; Nagel *et al.*, 1997), and OCT2 (Busch *et al.*, 1998; Gründemann *et al.*, 1997; Gründemann *et al.*, 1998a; Okuda *et al.*, 1996). All three transporters are members of the amphiphilic solute facilitator (ASF) family of transport proteins (Schömig *et al.*, 1998) and accept catecholamines and MPP<sup>+</sup> as substrates (Gründemann *et al.*, 1999). EMT is expressed in many tissues, but OCT2 has been detected solely in kidney and brain, and OCT1 is confined to liver, kidney, and intestine. Collectively, we refer to these carriers as non-neuronal monoamine transporters.

The physiological and pathophysiological significance of non-neuronal monoamine transport is still fairly unclear. The first knock-outs in mouse emerge (Jonker *et al.*, 2001; Zwart *et al.*, 2001), but these may suffer from inherent methodological limitations. Important clues could come from the instant *in vivo* effects of potent and specific inhibitors in rat or mouse. Remarkably, in our experiments disprocynium24, the most efficacious known inhibitor of human EMT, turned out to be insufficiently potent on EMT from rat to be useful *in vivo*. We here report as likely cause a hitherto unknown activation mechanism for EMT from rat and mouse.

## Methods

### Construction of EMTr and EMTm expression vectors

For the cloning of EMTr, total RNA from Sprague–Dawley rat heart was prepared as described (Gründemann *et al.*, 1997). A cDNA covering the open reading frame entirely was generated by RT–PCR with *Pwo* Polymerase (Roche, Mannheim, Germany) and with primers 5'-GAG AGA CAG GTA CCG CCA CCA TGC CCA CGT TCG ACC (forward primer, *KpnI* site underlined; this primer introduces a perfect Kozak sequence) and 5'-GAG AGA GAT CTA GAC GGC GCC ACC TAG AGG AGG TGA (reverse primer, *XbaI* site underlined). The amplicon (1.8 kb) was cloned, completely sequenced and eventually assembled into the *KpnI* and *XbaI* sites of pcDNA3 (Invitrogen, NV Leek, The Netherlands) to yield pcDNA3EMTr. Sequencing of four independent clones uniformly revealed a base disparity with the published cDNA sequence (accession number AF055286), 761G>A, which gives rise to amino acid substitution G124S. The variant base was confirmed by PCR on genomic DNA from rat liver and subsequent cloning and sequencing of the generated amplicon. For further corroboration, we used in PCR on genomic DNA two primers that differed only at the ultimate 3'-base, i.e. 5'-TCC ACA TAG CGC CAG TCG CC or ...CG CT, as reverse primers in paired assays. With optimized conditions (forward primer as above, standard PCR (Schömig *et al.*, 1998) plus 5% DMSO, 32 cycles of 30 s 94°C, 1 min 70°C, 500 ng

*EcoRI*-digested genomic DNA from rat liver per assay) a product of expected size (0.40 kb) was generated solely with the primer ending in T.

A cDNA of EMTm (accession number AF078750) was kindly provided by Dr R. Zwart, Department of Molecular Genetics, The Netherlands Cancer Institute, Amsterdam, The Netherlands. The complete open reading frame was released from pCAGGS and inserted into pcDNA3 *via EcoRI*. The 5'-UTR was replaced by a perfect Kozak sequence. The junctions in the final construct, pcDNA3EMTm, were AAG CTTCGc acc atg ccc... (*HindIII* site of vector underlined; lower-case letters indicate original cDNA sequence) and ...agg tgg cgA TAC CGT CGA GAA TCC (*EcoRI* site of vector underlined).

### Cell culture and transfection

293 cells (ATCC CRL-1573), a transformed cell line derived from human embryonic kidney, were grown at 37°C in a humidified atmosphere (5% CO<sub>2</sub>) in plastic culture flasks (Falcon 3112, Becton Dickinson, Heidelberg, Germany). The medium was Dulbecco's Modified Eagle Medium (31885-023, Life Technologies, Eggenstein, Germany) supplemented with 10% foetal calf serum (Life Technologies). Medium was changed every 2–3 days and the culture was split every 7 days.

293 cells were transfected with supercoiled plasmid DNA by lipofection with the Tfx-50 reagent according to the protocol of the vendor (Promega, Mannheim, Germany). Stably transfected cells were then selected with G418 (Life Technologies) as described (Gründemann *et al.*, 1997). Expression of EMTr and EMTm was verified by RT–PCR and functional characterization.

### Transport assays

For measurement of uptake of radiolabeled solutes, cells were grown in surface culture on 60 mm polystyrol dishes (Nunc 150288, Nunc, Wiesbaden, Germany) precoated with 0.1 g l<sup>-1</sup> poly-L-ornithine in 0.15 mol l<sup>-1</sup> boric acid-NaOH, pH 8.4. Cells were used for uptake experiments at a confluence of at least 70%.

Uptake was measured at 37°C. After preincubation for at least 20 min in 4 ml of Na<sup>+</sup> buffer (125 mmol l<sup>-1</sup> NaCl, 25 mmol l<sup>-1</sup> HEPES-NaOH pH 7.4, 5.6 mmol l<sup>-1</sup> (+)glucose, 4.8 mmol l<sup>-1</sup> KCl, 1.2 mmol l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 1.2 mmol l<sup>-1</sup> CaCl<sub>2</sub>, 1.2 mmol l<sup>-1</sup> MgSO<sub>4</sub>), the buffer was replaced with 3 ml of <sup>3</sup>H-labelled substrate (at 100 nmol l<sup>-1</sup> if not noted otherwise) and inhibitor as indicated in Na<sup>+</sup> buffer. NaCl was iso-osmotically reduced if concentration of inhibitor exceeded 1 mmol l<sup>-1</sup>. In some experiments, K<sup>+</sup> buffer was used instead of Na<sup>+</sup> buffer K<sup>+</sup> is identical to Na<sup>+</sup> buffer except that NaCl and HEPES-NaOH were completely replaced by KCl and HEPES-KOH, respectively. Incubation was stopped (after 1 min if not noted otherwise) by rinsing the cells four times with each 4 ml ice-cold Na<sup>+</sup> buffer. Subsequently, the cells were solubilized with 0.1% v v<sup>-1</sup> Triton X-100 in 5 mmol l<sup>-1</sup> TRIS-HCl pH 7.4, and radioactivity was determined by liquid scintillation counting. In experiments with noradrenaline, 1 mmol l<sup>-1</sup> (+)ascorbic acid was present to prevent oxidation.

To avoid *trans*-stimulation effects with established or potential substrates, all inhibitors with a *K<sub>i</sub>* larger than

$1 \mu\text{mol l}^{-1}$  were absent during preincubation. By contrast, potent inhibitors ( $K_i$  smaller than  $1 \mu\text{mol l}^{-1}$ ) were present during both the preincubation and uptake periods. This is necessary to achieve equilibrium binding with effective inhibitors – which are used at very low concentrations – and hence realize their full potency. These high-affinity inhibitors are at best transported very slowly and thus do not cause *trans*-stimulation.

#### Protein determination

Protein was measured by a modification of the Bradford Method (Zor & Selinger, 1996) with bovine serum albumin as standard.

#### Calculations and statistics

Analysis of the time course of substrate accumulation was based on a cone-compartment model as described earlier (Russ *et al.*, 1992). Saturation curves were analysed as described (Schömig *et al.*, 1993).

For EMTh, all  $K_T$ -values were calculated by non-linear regression fitting to the standard model (assuming a Hill coefficient of 1):  $v_i/v_0 = 1/(1 + I/IC_{50})$  with  $v_i$ : specific uptake velocity in the presence of inhibitor,  $v_0$ : specific uptake velocity in the absence of inhibitor, and  $I$ : concentration of inhibitor.  $IC_{50}$ -values are identical with  $K_i$  values, since non-saturating substrate concentrations were used (Cheng & Prusoff, 1973). For EMTr and EMTm, the general activation model according to Figure 9 can be factorized into  $v_i/v_0 = (a_1 + a_2 * I)/(a_1 + a_3 * I + a_4 * I^2)$ ; parameters  $a_1$ ,  $a_2$ ,  $a_3$ , and  $a_4$  are functions of dissociation constants, rate constants, and substrate concentration. Division by  $a_1$  yields  $v_i/v_0 = (1 + a * I)/(1 + b * I + c * I^2)$  with phenomenological constants  $a$ ,  $b$ , and  $c$ . This equation was used for non-linear regression.

Fitted parameters such as  $K_m$ - and  $K_T$ -values are given as geometric mean with 95% confidence interval. Arithmetic means are given with s.e.mean. In the figures, error bars smaller than symbols are not shown.

#### Drugs

Radiotracers: 1-methyl-4-phenylpyridinium iodide (H-3, 2200 Bq pmol<sup>-1</sup>; ART-150, ARC, St. Louis, U.S.A.), (–)noradrenaline (norepinephrine) (H-3, 381 Bq pmol<sup>-1</sup>; NET-337, NEN, Zaventem, Belgium), and cimetidine (H-3, 455 Bq pmol<sup>-1</sup>; TK615, Amersham Pharmacia, Freiburg, Germany).

Unlabelled compounds: BU-224 and BU-226 (0725 and 1091, Tocris, Bristol, U.K.), cimetidine (28541-2, Aldrich, Steinheim, Germany), clobenpropit (0752, Tocris), corticosterone (C-2505, Sigma, Deisenhofen, Germany), decynium22 (1,1'-diethyl-2,2'-cyanine iodide, 32376-4, Aldrich), DMPBI (1,3-dimethyl-2-phenyl-3H-benzimidazol-1-ium chloride, S136077, Sigma-Aldrich library of rare chemicals), histamine dihydrochloride (4370, Merck, Darmstadt, Germany), immepip (0932, Tocris), MPP<sup>+</sup> (1-methyl-4-phenylpyridinium iodide, D-048, RBI, Natick, MA, U.S.A.), (–)noradrenaline hydrochloride (74480, Fluka, Buchs, Switzerland), papaverine (P-3510, Sigma), phentolamine hydrochloride (CIBA, Summit, NJ, U.S.A.), thioperamide (T-123, RBI), and trimazosine hydro-

chloride (Lappe Arzneimittel, Berg. Gladb., Germany). Disprocynium24 (1,1'-diisopropyl-2,4-cyanine iodide) was synthesized as described previously (Russ *et al.*, 1993). All other chemicals were of analytical grade.

## Results

#### *Cloning and functional expression of EMTr in 293 cells*

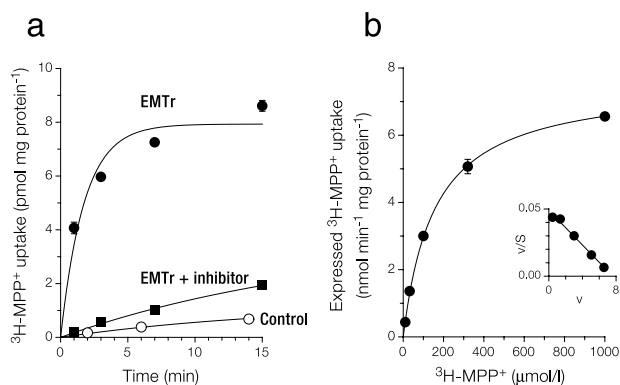
Based on the published cDNA sequence (Kekuda *et al.*, 1998), we have cloned EMTr from rat heart by RT-PCR with specific primers. Sequencing and additional testing (see Methods for details) revealed a base disparity which gives rise to amino acid substitution G124S. Presumably, this amino acid exchange has no major functional consequence, since EMTm, the orthologue carrier from mouse, contains a glycine at the equivalent position and is functionally similar to our EMTr clone (see below). For functional expression, the cDNA of EMTr was inserted into the eukaryotic expression vector pcDNA3. The resulting plasmid, pcDNA3EMTr, was used to stably transfect 293 cells, our standard cell line for heterologous expression of non-neuronal monoamine transporters (Gründemann *et al.*, 1999). Non-specific uptake was measured with control cells that were stably transfected with empty vector.

Functional expression of EMTr was verified in uptake experiments with adherent cells in plastic dishes. A detailed analysis of the time course of uptake of <sup>3</sup>H-MPP<sup>+</sup> into pcDNA3EMTr-transfected cells (Figure 1a) revealed rate constants for inwardly ( $k_{in}$ ) and outwardly ( $k_{out}$ ) directed MPP<sup>+</sup> fluxes of  $45 \pm 7 \mu\text{l min}^{-1} \text{mg protein}^{-1}$  and  $0.6 \pm 0.1 \text{ min}^{-1}$ , respectively. The uptake at equilibrium ( $A_{max}$ ) amounted to  $7.9 \pm 0.3 \text{ pmol mg protein}^{-1}$ . Based on an intracellular water space of  $6.7 \mu\text{l mg protein}^{-1}$  (Martel *et al.*, 1996) and a conjectured transfection efficiency of 100%, at equilibrium a 12 fold accumulation of MPP<sup>+</sup> relative to medium can be estimated. MPP<sup>+</sup> uptake was largely, but not completely inhibited by  $10 \mu\text{mol l}^{-1}$  disprocynium24. An uptake period of 1 min was chosen for subsequent experiments to approximate initial rates of transport.

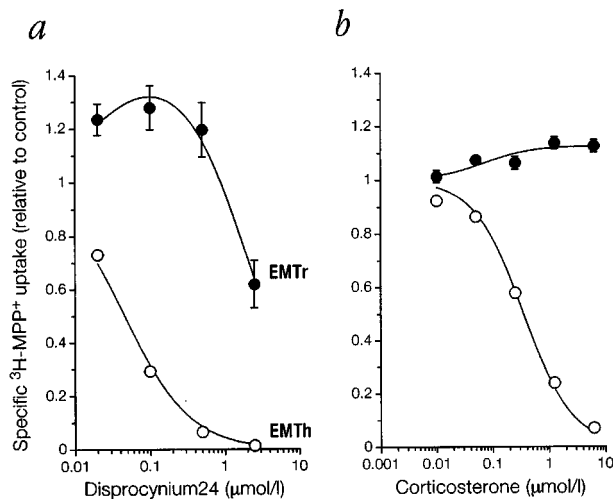
Expressed uptake, i.e. total uptake minus total uptake into control cells, of MPP<sup>+</sup> was saturable (Figure 1b), with an apparent Michaelis-Menten constant,  $K_m$ , of 151 (95% confidence interval, 133–172)  $\mu\text{mol l}^{-1}$  and a maximal uptake rate,  $V_{max}$  of  $7.5 \pm 0.1 \text{ nmol min}^{-1} \text{mg protein}^{-1}$ . The Eadie-Hofstee plot is compatible with a single uptake mechanism.

#### *Inhibitors of EMTh are less potent on EMTr*

Inhibition of specific <sup>3</sup>H-MPP<sup>+</sup> uptake by disprocynium24, the most potent inhibitor available for the human EMT (Russ *et al.*, 1993), was quantitatively examined for EMTr and EMTh, both expressed in stably transfected 293 cell lines (Figure 2a). Specific uptake was defined as that fraction of total uptake that was sensitive to  $10 \mu\text{mol l}^{-1}$  disprocynium24. The affinity for disprocynium24 of EMTr (extrapolated  $IC_{50} \approx 4 \mu\text{mol l}^{-1}$ ) appeared to be almost two orders of magnitude lower than that of EMTh ( $K_i = 46$  (42–51)  $\text{nmol l}^{-1}$ ). A large difference was also observed for corticosterone (Figure 2b), a well-established and relatively



**Figure 1** Characterization of 293 cells expressing EMTr in Na<sup>+</sup> buffer. (a) Time course of MPP<sup>+</sup> uptake. Cells grown in dishes were incubated at 37°C with 0.1 μmol l<sup>-1</sup> MPP<sup>+</sup>. Shown is mean ± s.e.mean ( $n=3$ ). Shown are control cells (stably transfected with vector pcDNA3,  $k_{in}=0.74\pm 0.07 \mu\text{l min}^{-1} \text{mg protein}^{-1}$ ,  $k_{out}=0.06\pm 0.01 \text{min}^{-1}$ ), EMTr cells (see main text for kinetic constants) and EMTr cells in the presence of 10 μmol l<sup>-1</sup> disprocynium24 (also during preincubation;  $k_{in}=1.8\pm 0.2 \mu\text{l min}^{-1} \text{mg protein}^{-1}$ ,  $k_{out}=0.05\pm 0.02 \text{min}^{-1}$ ). (b) Saturation of expressed uptake of MPP<sup>+</sup>. An uptake period of 1 min was chosen to approximate initial rates of transport. Shown is mean ± s.e.mean ( $n=3$ ). Expressed uptake was calculated by subtraction from total uptake of uptake into cells transfected with vector pcDNA3 alone. This control uptake increased linearly with MPP<sup>+</sup> concentration, slope = 0.66 μl min<sup>-1</sup> mg protein<sup>-1</sup>. The graph shown was obtained by fitting  $v = V_{max} * S / (K_m + S)$  by non-linear regression, with  $v$ : uptake velocity, and  $S$ : substrate concentration. Inset: Eadie-Hofstee transformation. With our model of activation (see Figure 9), the corresponding equation is  $v = V_{max} * S * (1 + g * S / K_d) / (K_m + S * (1 + S / K_d))$ . After fitting by non-linear regression, the graph coincides with the one shown.



**Figure 2** Inhibition by (a) disprocynium24 or (b) corticosterone of specific MPP<sup>+</sup> uptake into 293 cells expressing EMTr or EMTh in Na<sup>+</sup> buffer. Following preincubation for 20 min in the presence of inhibitor, uptake into 293 cells expressing EMTh or EMTr was measured for 1 min to approximate initial rates of transport. Shown as a function of inhibitor concentration is mean ± s.e.mean ( $n=3$ ) of the specific uptake of <sup>3</sup>H-MPP<sup>+</sup> (0.1 μmol l<sup>-1</sup>) relative to control (no inhibitor present). Specific uptake was defined as that fraction of total uptake that was sensitive to 10 μmol l<sup>-1</sup> disprocynium24. The graphs shown result from fitting to different models for EMTh and EMTr as detailed in Methods.

potent inhibitor of EMTh ( $K_i=0.35$  (0.31–0.40) μmol l<sup>-1</sup> in this work), but much less so for EMTr ( $IC_{50}>10 \mu\text{mol l}^{-1}$ ). In additional experiments, estimated  $IC_{50}$ -values of EMTr for decynium22 (6.4 (1.3–33) μmol l<sup>-1</sup>) and 3-O-methylisoprenaline (64 (29–144) μmol l<sup>-1</sup>) were higher than  $K_i$ -values of EMTh by a factor of 200 and 22, respectively.

In an effort to identify a powerful inhibitor for EMTr, we tested in parallel a range of compounds for inhibitory potency on EMTr and EMTh. Since histamine is an excellent substrate for EMTh (Gründemann *et al.*, 1999), some compounds were histamine and imidazoline (Farsang & Kapocsi, 1999) receptor antagonists. Most compounds were by a factor of 10 to 100 more potent inhibitors on EMTh than on EMTr, and only very few such as e.g. BU-224 or BU-226 were about equally effective (Table 1). None of the tested inhibitors had a  $K_i$  below 100 nmol l<sup>-1</sup>, as would be desirable for an inhibitor to be effective *in vivo* (Schömig & Schönfeld, 1990). Surprisingly, for EMTr we noticed with some compounds, e.g. disprocynium24 (Figure 2a), clobenpropit, imipip, papaverine, thioperamide, and DMPBI (Figure 3a), a concentration-dependent stimulation of <sup>3</sup>H-MPP<sup>+</sup> uptake in the presence of inhibitor up to a factor of 1.5 relative to control. This activation was not seen with EMTh.

#### Mechanism of activation

The stimulation by compound DMPBI of EMTr-mediated <sup>3</sup>H-MPP<sup>+</sup> uptake was analysed in more detail. Transport of cationic substrates by non-neuronal monoamine transporters is markedly affected by membrane potential. In order to rule out activation of transport by hyperpolarization of plasma membrane, we measured uptake in a buffer with K<sup>+</sup> in place of Na<sup>+</sup> ions. This manoeuvre depolarizes the plasma membrane and clamps the potential essentially to 0 mV (Schömig *et al.*, 1992). Under this condition the activation was not abolished but enhanced (Figure 3b). Therefore, hyperpolarization is not a plausible explanation for the observed activation. For most subsequent experiments K<sup>+</sup> buffer was used to avoid any influence of membrane potential.

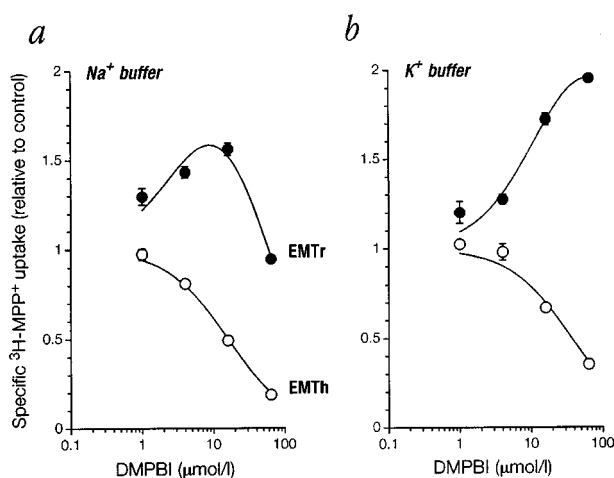
We next examined whether the activation was caused by *trans*-stimulation, i.e. acceleration of uptake by counter-transport of intracellular substrate. Cells expressing EMTr were preincubated for 24 min in K<sup>+</sup> buffer with 1 mmol l<sup>-1</sup> of unlabelled MPP<sup>+</sup>. Controls were incubated in K<sup>+</sup> buffer alone. Following thorough washing, uptake of <sup>3</sup>H-MPP<sup>+</sup> was measured as usual. As expected, EMTr cells preloaded with MPP<sup>+</sup> showed a marked increase in <sup>3</sup>H-MPP<sup>+</sup> uptake versus control cells (Figure 4). On the basis of specific uptake, this *trans*-stimulation amounts to a factor of  $3.7\pm 0.2$ . Nonetheless, in the presence of DMPBI the uptake was stimulated further (factor =  $1.5\pm 0.1$ ), similar to cells not preloaded with MPP<sup>+</sup> (factor =  $1.8\pm 0.1$ ). If DMPBI was involved in *trans*-stimulation, it would have to compete intracellularly with a large excess of MPP<sup>+</sup> and relative activation should decrease strongly. Thus, *trans*-stimulation and activation are probably independent mechanisms, and activation does not originate from *trans*-stimulation.

<sup>3</sup>H-MPP<sup>+</sup> uptake was also higher than control when cimetidine was used as inhibitor (Figure 5a). However, when <sup>3</sup>H-cimetidine was used as substrate, unlabelled cimetidine did

**Table 1** Inhibition by various compounds of specific  $^3\text{H-MPP}^+$  uptake into stably transfected 293 cells expressing either EMTh or EMTr

Compound	EMTh		EMTr		$c_i$ range ( $\mu\text{mol l}^{-1}$ )
	$K_i$ ( $\mu\text{mol l}^{-1}$ )	95% c.i.	$K_i$ ( $\mu\text{mol l}^{-1}$ )	95% c.i.	
Papaverine	0.53	0.44, 0.64	35	13, 99	0.3, 37.5
Trimazosine	1.1	0.88, 1.4	96	42, 220	0.3, 37.5
Clobenpropit	1.2	0.99, 1.4	74	14, 390	0.1, 21.6
Thioperamide	3.2	2.2, 4.6	33	12, 95	0.2, 43.2
Phentolamine	4.6	4.2, 5.1	140	91, 200	1, 125
DMPBI	16	14, 18	>64		1, 64
BU-226	44	30, 64	40	26, 60	0.2, 43.2
BU-224	59	37, 95	59	40, 89	0.2, 43.2
Histamine	160	130, 190	1000	670, 1600	30, 3750
Immepip	>21.6		>21.6		0.1, 21.6

Given is the  $K_i$  with the corresponding 95% confidence interval ( $n=12$ ) and the tested range of concentration of inhibitor. All  $K_i$ -values were calculated as described in Methods for EMTh. The concentration of  $^3\text{H-MPP}^+$  was  $0.1 \mu\text{mol l}^{-1}$ .



**Figure 3** Inhibition by compound DMPBI of specific  $\text{MPP}^+$  uptake into 293 cells expressing EMTr or EMTh in (a) standard buffer or (b) high potassium buffer. In  $\text{K}^+$  buffer, all  $\text{Na}^+$  ions were replaced by  $\text{K}^+$  to depolarize and clamp membrane potential. For both conditions, cells were preincubated without inhibitor in the respective uptake buffers for 24 min. For EMTr, control uptake in  $\text{K}^+$  buffer was 44% of uptake in  $\text{Na}^+$  buffer, and non-specific uptake amounted to 5% of total uptake in  $\text{Na}^+$  buffer and 7% in  $\text{K}^+$  buffer. For further details see legend to Figure 2.

not stimulate uptake above control level. Hence, activation strikingly depends on the structure of the monitored substrate. In accordance with this concept,  $\text{MPP}^+$  as inhibitor did not stimulate uptake of  $^3\text{H-cimetidine}$  (Figure 5b), but markedly activated uptake of  $^3\text{H-MPP}^+$  (Figure 6).

The differential activation seen for uptake of  $^3\text{H-cimetidine}$  and  $^3\text{H-MPP}^+$  could relate to the use of inhibitors that are transported themselves, i.e. cimetidine and  $\text{MPP}^+$ . We therefore tested activation of uptake by a non-transported inhibitor, disprocynium24 (Figure 7). Again, uptake of  $^3\text{H-MPP}^+$  was clearly stimulated above control level, while uptake of  $^3\text{H-cimetidine}$  was not. Stimulation of uptake of  $^3\text{H-noradrenaline}$  was intermediate. Note that  $\text{IC}_{50}$ -values (disprocynium24 concentration at uptake relative to control = 0.5) are considerably dissimilar for different substrates.

To confirm that the mechanism of activation also operates under physiological conditions, we measured stimulation of uptake of  $^3\text{H-MPP}^+$  by unlabelled  $\text{MPP}^+$  in  $\text{Na}^+$  buffer

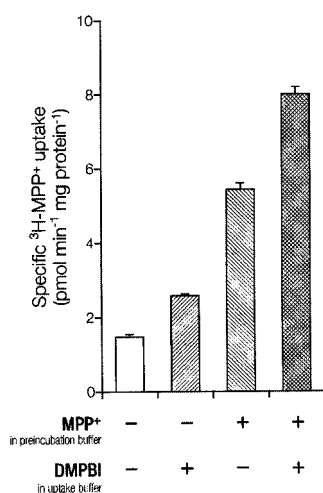
(Figure 8). Compared to uptake in  $\text{K}^+$  buffer the effect is somewhat smaller, but openly evident. This agrees well with the data presented in Figure 3.

For verification of results, we obtained the cDNA of the EMT orthologue from mouse, which is highly similar to EMTr (98.7% amino acid sequence identity). Functional expression of the EMTm cDNA in 293 cells was performed as described above. In excellent agreement with the results for EMTr,  $^3\text{H-MPP}^+$  uptake mediated by EMTm was strongly activated by unlabelled  $\text{MPP}^+$  (Figure 6). Also, the  $K_i$ -values of EMTm for disprocynium24 ( $0.29$  ( $0.18$ – $0.45$ )  $\mu\text{mol l}^{-1}$ ) and corticosterone ( $11$  ( $4.4$ – $26$ )  $\mu\text{mol l}^{-1}$ ) were similar to the above values for EMTr.

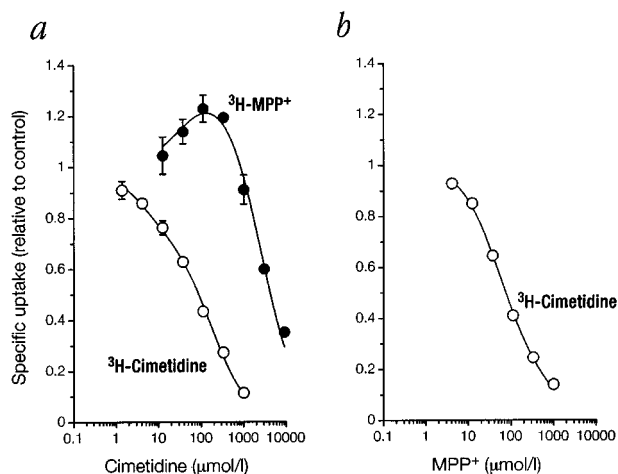
## Discussion

The first hint that EMT from rat is markedly different in kinetic terms from other non-neuronal monoamine transporters and even from the human orthologue EMTh came from the  $K_m$ -values for  $\text{MPP}^+$ . Whereas OCT2 and OCT1 both from human and rat and EMT from human display very similar affinities ( $10$ – $20 \mu\text{mol l}^{-1}$ ) (Gründemann *et al.*, 1999; manuscript in preparation), EMT from rat was reported with a  $K_m$  of  $90$ – $140 \mu\text{mol l}^{-1}$  in HRPE cells (Wu *et al.*, 1998). Our present results confirm this difference (Figure 1b). We also report here that known highly potent EMTh inhibitors such as disprocynium24, decynium22, and corticosterone are much less potent on EMT from rat (Figure 2) and mouse. At the same time, there is a pressing need for potent inhibitors in rat or mouse in order to promote understanding of physiological function and pharmacology of EMT in these principal mammalian model organisms. Thus far, our efforts to identify a truly potent inhibitor for EMTr have not been successful. However, an unexpected finding may explain the peculiar characteristics of EMTr. In this work, we report that inhibitor may stimulate EMTr-mediated transport above control level, an unusual and hitherto unrecognized phenomenon which we refer to as activation.

In order to verify this observation, we have sought for rat cell lines that natively express EMTr. With RT-PCR, none of the tested lines (A10, C6, RINm5F, RGE, NRK-52E, and PC12) was positive (not shown), which is quite in contrast to EMTh which has been detected in many cell lines. However,

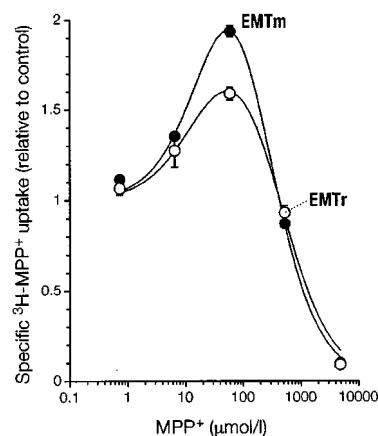


**Figure 4** *Trans*-stimulation and activation of MPP<sup>+</sup> uptake into 293 cells expressing EMTr in K<sup>+</sup> buffer. Shown is mean ± s.e.mean (*n* = 3) of specific uptake. Specific uptake is total uptake minus non-specific uptake. Non-specific uptake (0.58 ± 0.01 pmol min<sup>-1</sup> mg protein<sup>-1</sup>) was measured in parallel assays with 10 μmol l<sup>-1</sup> disprocynium24 both in preincubation and uptake buffers to inhibit EMTr. Stably transfected cells grown in dishes were preincubated for 24 min in K<sup>+</sup> buffer (control), supplemented where indicated with 1 mmol l<sup>-1</sup> of unlabelled MPP<sup>+</sup> for loading. After washing three times with each 4 ml ice-cold buffer to remove substrate from extracellular space, uptake of <sup>3</sup>H-MPP<sup>+</sup> (0.1 μmol l<sup>-1</sup>, 1 min) in K<sup>+</sup> buffer was measured. Parallel assays were performed with the addition of 50 μmol l<sup>-1</sup> unlabelled DMPBI solely to uptake buffer.

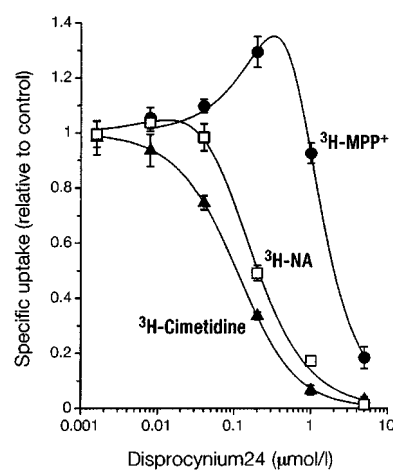


**Figure 5** Inhibition by (a) cimetidine or (b) MPP<sup>+</sup> of uptake into 293 cells expressing EMTr in K<sup>+</sup> buffer. Shown as a function of inhibitor concentration is mean ± s.e.mean (*n* = 3) of specific uptake relative to control of <sup>3</sup>H-MPP<sup>+</sup> or <sup>3</sup>H-cimetidine (each at 0.1 μmol l<sup>-1</sup>) as indicated. Cells were preincubated without inhibitor for 24 min. For further details see legend to Figure 2. With the special case of substrate equal to inhibitor and with the (unproved) assumption of complete symmetry (i.e.  $K_i = K_s$ ,  $K_a = K_b = K_c = K_d$ , and  $e = f = g$ ; see Figure 9), the general model of activation simplifies to  $v_i/v_0 = (K_s + S + S^2/K_d) * (K_d + g * S + 2 * g * I) / ((K_d + g * S) * (K_s + S + I + 1 / K_d * (S + I)^2))$ . The parameters extracted for cimetidine by non-linear regression are  $K_s = 4.0$  (1.4–12) μmol l<sup>-1</sup>,  $K_d = 180$  (120–260) μmol l<sup>-1</sup> and  $g = 16$  (6.2–40).

the almost identical orthologue from mouse, EMTm, showed features very similar to EMTr, providing ample corroboration of our results. Note that whereas the concentration of



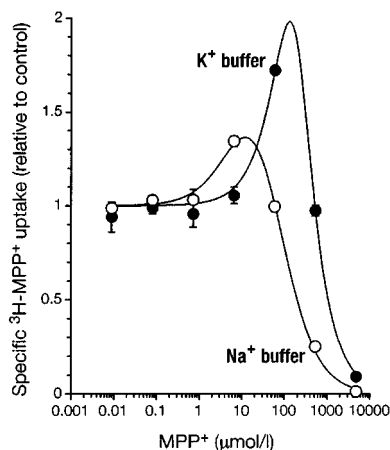
**Figure 6** Comparison of EMT from rat and mouse. Shown as a function of inhibitor concentration is mean ± s.e.mean (*n* = 3) of specific uptake relative to control of <sup>3</sup>H-MPP<sup>+</sup> (0.1 μmol l<sup>-1</sup>) in K<sup>+</sup> buffer into 293 cells expressing EMTr or EMTm as indicated. Cells were preincubated without inhibitor for 24 min. For further details see legend to Figure 2. Parameters fitted as described in legend to Figure 5 are  $K_s = 26$  (18–37) (EMTm) and 17 (6.5–42) μmol l<sup>-1</sup> (EMTr),  $K_d = 230$  (190–280) and 440 (300–660) μmol l<sup>-1</sup>, and  $g = 13$  (8.3–20) and 27 (9.0–79).



**Figure 7** Inhibition by disprocynium24 of uptake of cimetidine, noradrenaline (NA), or MPP<sup>+</sup> into 293 cells expressing EMTr in K<sup>+</sup> buffer. Shown as a function of inhibitor concentration is mean ± s.e.mean (*n* = 3) of specific uptake relative to control of labelled substrate (each at 0.1 μmol l<sup>-1</sup>) as indicated. Cells were preincubated with inhibitor for 24 min. For further details see legend to Figure 2.

unlabelled MPP<sup>+</sup> giving maximum stimulation in a given buffer was equal for both transporters (Figure 6) and invariable between experiments, the actual factor of maximum stimulation varied (not shown).

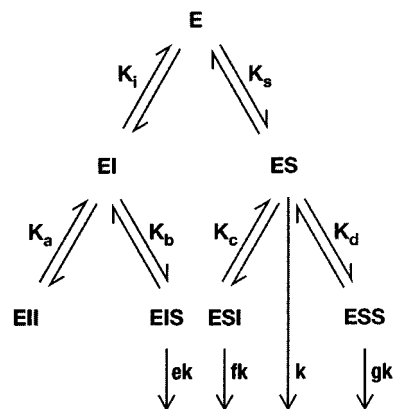
Our experiments demonstrate that activation is affected, but not caused by changes in membrane potential (Figure 3). The stronger activation (relative to control) in K<sup>+</sup> buffer versus Na<sup>+</sup> buffer may originate from attenuated binding of inhibitor due to membrane potential depolarization. In other words, in K<sup>+</sup> buffer, activation is more pronounced since the opposing inhibition of uptake is shifted to higher concentrations of inhibitor. Activation by inadvertent *trans*-stimulation (also known as accelerated exchange) has been reported



**Figure 8** Comparison of activation of  $MPP^+$  uptake in standard buffer ( $Na^+$  buffer) or  $K^+$  buffer. Shown as a function of inhibitor concentration is mean  $\pm$  s.e. mean ( $n=3$ ) of specific uptake of  $^3H$ - $MPP^+$  ( $1 \text{ nmol l}^{-1}$ ) relative to control into 293 cells expressing EMTr. For both conditions, cells were preincubated in the respective buffers for 24 min without inhibitor. For further details see legend to Figure 2.

(Deves & Krupka, 1987), but this mechanism depends on rapid passive diffusion of a substrate across the plasma membrane. In our case, disprocynium24 clearly evokes activation (Figures 2a and 7), but it should not be transported by EMTr at all. This and the experiment shown in Figure 4 suggest that activation is not based on *trans*-stimulation. Also, the experiments shown in Figures 5a and 7 effectively exclude the involvement of a receptor-mediated signalling pathway, since any receptor ligand would affect the transporter indirectly, irrespective of the substrate (compare  $MPP^+$  and cimetidine). For the same reason and since activation is immediate, i.e. without any preincubation of cells in the presence of inhibitor, an inhibitor-induced recruitment of a transporter reserve (Zhang & Ismail-Beigi, 1998) is implausible. An apparent increase in uptake activity could also result from protection of the labelled substrate against some extracellular inactivation with increasing concentrations of cold substrate or competitor. However, this explanation does not apply here, since (1) EMTh should be affected to the same extent as EMTr, and (2) e.g.  $MPP^+$  is highly resistant to metabolism.

Instead, for an explanation of activation of EMTr and EMTm, we propose a model that assumes the interaction of two binding sites for substrate or inhibitor (Figure 9). Note that the familiar cooperativity-based Hill model for multisite inhibition (Segel, 1975) is unsuitable, since uptake in the presence of inhibitor will never exceed control uptake. Compared to two sites that operate independently, our model of activation suggests that inhibitor bound to the first site actually diminishes binding, but accelerates turnover of substrate at the second site (not shown). Thus, with  $MPP^+$  at moderate inhibitor concentrations (Figure 6), the gain in speed dominates over loss of affinity and activation becomes apparent. At high inhibitor concentrations, uptake eventually declines because two molecules of inhibitor bind to the transporter. By contrast, with cimetidine as substrate (Figure 5), no activation is apparent. This is intriguing, since cimetidine (I) is rather a good substrate for EMTh, with a



**Figure 9** Kinetic model for the activation of EMTr and EMTm. The model assumes two binding sites for inhibitor (I) or radiolabeled substrate (S) per transporter entity (E). For a full mathematical description of complex formation and substrate turnover, 10 parameters are required, i.e. equilibrium dissociation constants ( $K_s$ ,  $K_i$ ,  $K_a$ ,  $K_b$ ,  $K_c$ , and  $K_d$ ), a rate constant ( $k$ ) and relative factors ( $e$ ,  $f$ , and  $g$ ).

transport efficiency of 10% relative to  $MPP^+$  (Gründemann *et al.*, 1999), and (2) has an affinity for EMTh similar to  $MPP^+$ . Apparently, the structure of cimetidine does not sufficiently promote stimulation of turnover.

Since our model would require the fitting of 10 dissociation or rate constants, it is not possible, in general, to extract with confidence these parameters from the experiments reported in this article. However, the model achieves a good fit of relative uptake as a function of inhibitor concentration with three aggregate parameters (see Methods for equation). With the assumption of two kinetically indistinguishable interacting sites, the model simplifies considerably if substrate and inhibitor are identical (except for the radiolabel on the substrate). Thus, we have been able to calculate intrinsic  $K_i$  values for  $MPP^+$  and cimetidine (see legend to Figure 5). For  $MPP^+$ , the fitted values of EMTr ( $17 \mu\text{mol l}^{-1}$ ) and EMTm ( $26 \mu\text{mol l}^{-1}$ ) agree very well with the corresponding value of EMTh ( $24 \mu\text{mol l}^{-1}$  (Russ *et al.*, 1992)). For cimetidine, the extracted  $K_i$  of EMTr ( $4.0 \mu\text{mol l}^{-1}$ ) is somewhat lower than expected from experiments with EMTh ( $38 \mu\text{mol l}^{-1}$  (Gründemann *et al.*, 1998b)).

The extent of activation may depend on substrate affinity. Substrates of EMT with high affinity but slow turnover like  $MPP^+$  (Gründemann *et al.*, 1999) could strongly benefit from stimulation of turnover (structure permitting, see cimetidine), whereas substrates with low affinity but high  $V_{\text{max}}$  such as noradrenaline are less likely to show this effect (*cf.* Figure 7). It is evident from our study that no conclusions can be drawn from the observation of activation whether an inhibitor is actually transported, since both transported (e.g.  $MPP^+$ , cimetidine) and non-transported inhibitors (disprocynium24, corticosterone) elicited activation.

It is a generally accepted pharmacological principle that a transporter protein has a definite affinity ( $K_i$ ) for an inhibitor irrespective of substrate. However, with EMTr we demonstrate that inhibition potency if determined as usual from the  $IC_{50}$ -values depends very much on substrate (Figures 5 and 7). Clearly, the useful pharmacological concept to compare and identify carrier proteins based on  $IC_{50}$  values may fail with EMTr.

In rat brain a transport mechanism for catecholamines has been described that matches the extraneuronal monoamine transporter (uptake<sub>2</sub>) in all aspects except for the inhibition profile (Hendley *et al.*, 1970; Wilson *et al.*, 1988). IC<sub>50</sub>-values (1) are relatively high for e.g., corticosterone and 3-O-methylisoprenaline and (2) do not correlate closely with values from heart, a reference organ for uptake<sub>2</sub>. In experiments with rat heart, with isoprenaline or noradrenaline as substrates, there are no obvious signs of activation (Grohmann & Trendelenburg, 1984). We may now suggest that the protein behind the unidentified mechanism in rat brain is EMTr, and that activation causes the unusual inhibition pattern, since (1) activation increases IC<sub>50</sub>-values and (2) the extent of activation (and hence the shift of IC<sub>50</sub>-values) will not be uniform for different inhibitors. Further work is necessary to clarify why activation is apparently inoperative in rat heart. Perhaps activation is proportional to local transporter density or is blocked by other proteins (see below).

What would be the physiological purpose of activation? In contrast to cooperative proteins such as haemoglobin or aspartate transcarbamoylase, the saturation curve of EMTr is not plainly sigmoidal (Figure 1b), i.e. the fit to the hyperbolic Michaelis-Menten equation even at low substrate concentrations is as good as the fit to an equation based on our more complicated model (see legend to Figure 1b). Thus, we feel that activation of EMTr does not serve regulation of activity in response to substrate concentration. However, a suitable endogenous compound could conceivably evoke stimulation of transporter activity. Alternatively, we speculate that in rodents activation may keep the transporter working as it

encounters inhibitors. Much higher concentrations of blocking substances are necessary to shut the transporter down. This relates to the notion that rodents tolerate higher levels of xenobiotics than man.

The primary structures of EMTr and EMTh are very similar (87.1% amino acid identity), yet only EMTr shows activation. It will be interesting to determine the underlying molecular details. So far, it is not possible to tell whether the two postulated binding sites reside on a single transporter molecule (intramolecular activation) or whether a dimer of single-site transporters is in operation (intermolecular activation). The later case would open the possibility for the formation of heterodimers with novel functional characteristics within the ASF family, an aspect neglected so far in heterologous expression of cloned transporters.

In conclusion, it should be difficult to evaluate the role of the extraneuronal monoamine transporter in the rat or mouse model by the use of pharmacological inhibitors, since EMTr and EMTm may be much more resistant than the human orthologue. We have uncovered a mechanism of activation to explain this difference. The extent of activation critically depends on the chemical structure of the monitored substrate. Kinetic modelling suggests that activation is based on the interaction of two substrate binding sites per transporter entity.

Supported by Deutsche Forschungsgemeinschaft (SCHO 373/4-1 and FOR 302/2 B2). We thank Katarzyna Baran and Anke Ripperger for skillful technical assistance, and R. Zwart for providing the cDNA of EMTm.

## References

- BREIDERT, T., SPITZENBERGER, F., GRÜNDEMANN, D. & SCHÖMIG, E. (1998). Catecholamine transport by the organic cation transporter type 1 (OCT1). *Br. J. Pharmacol.*, **125**, 218–224.
- BUSCH, A.E., KARBACH, U., MISKA, D., GORBOULEV, V., AKHOUNDOVA, A., VOLK, C., ARNDT, P., ULZHEIMER, J.C., SONNERS, M.S., BAUMANN, C., WALDEGGER, S., LANG, F. & KOEPEL, H. (1998). Human neurones express the polyspecific cation transporter hOCT2, which translocates monoamine neurotransmitters, amantadine, and memantine. *Mol. Pharmacol.*, **54**, 342–352.
- CHENG, Y.-C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K<sub>i</sub>) and the concentration of inhibitor which causes 50 per cent inhibition (I<sub>50</sub>) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099–3108.
- DEVES, R. & KRUPKA, R.M. (1987). Effects on transport of rapidly penetrating, competing substrates: activation and inhibition of the choline carrier in erythrocytes by imidazole. *J. Membr. Biol.*, **99**, 13–23.
- EISENHOFER, G., MCCARTY, R., PACAK, K., RUSS, H. & SCHÖMIG, E. (1996). Disprocyinium<sub>24</sub>, a novel inhibitor of the extraneuronal monoamine transporter, has potent effects on the inactivation of circulating noradrenaline and adrenaline in conscious rat. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **354**, 287–294.
- FARSANG, C. & KAPOCSI, J. (1999). Imidazoline receptors: from discovery to antihypertensive therapy (facts and doubts). *Brain Res. Bull.*, **49**, 317–331.
- GROHMANN, M. & TRENDELENBURG, U. (1984). The substrate specificity of uptake<sub>2</sub> in the rat heart. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **328**, 164–173.
- GRÜNDEMANN, D., BABIN-EBELL, J., MARTEL, F., ÖRDING, N., SCHMIDT, A. & SCHÖMIG, 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.
- GRÜNDEMANN, D., GORBOULEV, V., GAMBARYAN, S., VEYHL, M. & KOEPEL, H. (1994). Drug excretion mediated by a new prototype of polyspecific transporter. *Nature*, **372**, 549–552.
- GRÜNDEMANN, D., KÖSTER, S., KIEFER, N., BREIDERT, T., ENGELHARDT, M., SPITZENBERGER, F., OBERMÜLLER, N. & SCHÖMIG, E. (1998a). Transport of monoamine transmitters by the organic cation transporter type 2 (OCT2). *J. Biol. Chem.*, **273**, 30915–30920.
- GRÜNDEMANN, D., LIEBICH, G., KIEFER, N., KÖSTER, S. & SCHÖMIG, E. (1999). Selective substrates for non-neuronal monoamine transporters. *Mol. Pharmacol.*, **56**, 1–10.
- GRÜNDEMANN, D., SCHECHINGER, B., RAPPOLD, G.A. & SCHÖMIG, E. (1998b). Molecular identification of the corticosterone-sensitive extraneuronal monoamine transporter. *Nature Neurosci.*, **1**, 349–351.
- HENDLEY, E.D., TAYLOR, K.M. & SNYDER, S.H. (1970). 3H-normetanephrine uptake in rat brain slices. Relationship to extraneuronal accumulation of norepinephrine. *Eur. J. Pharmacol.*, **12**, 167–179.
- IVERSEN, L.L. (1965). The uptake of catecholamines at high perfusion concentrations in the rat isolated heart: a novel catecholamine uptake process. *Br. J. Pharmacol.*, **25**, 18–33.



- IVERSEN, L.L. & SALT, P.J. (1970). Inhibition of catecholamine uptake by steroids in the isolated rat heart. *Br. J. Pharmacol.*, **40**, 528–530.
- JONKER, J.W., WAGENAAR, E., MOL, C.A., BUITELAAR, M., KOEPESELL, 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). *Mol. Cell Biol.*, **21**, 5471–5477.
- KEKUDA, R., PRASAD, P.D., WU, X., WANG, H., FEI, Y.-J., LEIBACH, F.H. & GANAPATHY, V. (1998). Cloning and functional characterization of a potential-sensitive, polyspecific organic cation transporter (OCT3) most abundantly expressed in placenta. *J. Biol. Chem.*, **273**, 15971–15979.
- MARTEL, F., VETTER, T., RUSS, H., GRÜNDEMANN, D., AZEVEDO, I., KOEPESELL, H. & SCHÖMIG, E. (1996). Transport of small organic cations in the rat-liver - the role of the organic cation transporter OCT1. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **354**, 320–326.
- NAGEL, G., VOLK, C., FRIEDRICH, T., ULZHEIMER, J.C., BAMBERG, E. & KOEPESELL, H. (1997). A reevaluation of substrate specificity of the rat cation transporter rOCT1. *J. Biol. Chem.*, **272**, 31953–31956.
- OKUDA, M., SAITO, H., URAKAMI, Y., TAKANO, M. & INUI, K. (1996). cDNA cloning and functional expression of a novel rat kidney organic cation transporter, OCT2. *Biochem. Biophys. Res. Commun.*, **224**, 500–507.
- POVLOCK, S.L. & AMARA, S.G. (1997). The structure and function of norepinephrine, dopamine, and serotonin transporters. In *Neurotransmitter transporters. Structure, function, and regulation*. ed. Reith, M.E.A. pp. 1–28. Totowa: Humana Press.
- RUSS, H., ENGEL, W. & SCHÖMIG, E. (1993). Isocyanines and pseudoisocyanines as a novel class of potent noradrenalin transport inhibitors: synthesis, detection, and biological activity. *J. Medicinal Chem.*, **36**, 4208–4213.
- RUSS, H., GLIESE, M., SONNA, J. & SCHÖMIG, E. (1992). The extraneuronal transport mechanism for noradrenalin (uptake2) avidly transports 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>). *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **346**, 158–165.
- RUSS, H., STAUDT, K., MARTEL, F., GLIESE, M. & SCHÖMIG, E. (1996). The extraneuronal transporter for monoamine transmitters exists in cells derived from human central nervous system glia. *Eur. J. Neurosci.*, **8**, 1256–1264.
- SCHÖMIG, E., BABIN-EBELL, J. & RUSS, H. (1993). 1,1'-Diethyl-2,2'-cyanine (decynium22) potently inhibits the renal transport of organic cations. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **347**, 379–383.
- SCHÖMIG, E., BABIN-EBELL, J., RUSS, H. & TRENDELENBURG, U. (1992). The force driving the extraneuronal transport mechanism for catecholamines (uptake2). *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **345**, 437–443.
- SCHÖMIG, E. & SCHÖNFELD, C.L. (1990). Extraneuronal noradrenaline transport (uptake2) in a human cell line (Caki-1 cells). *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **341**, 404–410.
- SCHÖMIG, E., SPITZENBERGER, F., ENGELHARDT, M., MARTEL, F., ÖRDING, N. & GRÜNDEMANN, D. (1998). Molecular cloning and characterization of two novel transport proteins from rat kidney. *FEBS Lett.*, **425**, 79–86.
- SEGEL, I.H. (1975). *Enzyme kinetics*. New York: John Wiley & Sons.
- TRENDELENBURG, U. (1988). The extraneuronal uptake and metabolism of catecholamines. In *Catecholamines I*. eds. Trendelenburg, U. & Weiner, N. pp. 279–319. Berlin: Springer-Verlag.
- VERHAAGH, S., SCHWEIFER, N., BARLOW, D.P. & ZWART, R. (1999). Cloning of the mouse and human solute carrier 22a2 (Slc22a3/SLC22A3) identifies a conserved cluster of three organic cation transporters on mouse chromosome 17 and human 6q26-q27. *Genomics*, **55**, 209–218.
- WILSON, V.G., GROHMANN, M. & TRENDELENBURG, U. (1988). The uptake and O-methylation of 3H-(+/-)-isoprenaline in rat cerebral cortex slices. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **337**, 397–405.
- WU, X., HUANG, W., GANAPATHY, M.E., WANG, H., KEKUDA, R., CONWAY, S.J., LEIBACH, F.H. & GANAPATHY, V. (2000). Structure, function, and regional distribution of the organic cation transporter OCT3 in the kidney. *Am. J. Physiol.*, **279**, F449–F458.
- WU, X., KEKUDA, R., HUANG, W., FEI, Y.-J., LEIBACH, F.H., CHEN, J., CONWAY, S.J. & GANAPATHY, V. (1998). Identity of the organic cation transporter OCT3 as the extraneuronal monoamine transporter (uptake2) and evidence for the expression of the transporter in the brain. *J. Biol. Chem.*, **273**, 32776–32786.
- ZHANG, J.Z. & ISMAIL-BEIGI, F. (1998). Activation of Glut1 glucose transporter in human erythrocytes. *Arch. Biochem. Biophys.*, **356**, 86–92.
- ZOR, T. & SELINGER, Z. (1996). Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. *Anal. Biochem.*, **236**, 302–308.
- 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.

(Received June 5, 2002  
Accepted August 13, 2002)