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RESEARCH PAPER

Differential inhibitory effects of drugs acting at the noradrenaline and 5-hydroxytryptamine transporters in rat and human neocortical synaptosomes*

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Background and purpose: Although the amino acid sequences of rat and human 5-hydroxytryptamine (5-HT) and noradrenaline (NA) transporters (i.e. SERT and NET) are highly homologous, species differences exist in the inhibitory effects of drugs acting at these transporters. Therefore, comparison of the potencies of drugs acting at SERT and NET in native human and rat neocortex may serve to more accurately predict their clinical profile.
Experimental approach: Synaptosomes prepared from fresh human and rat neocortical tissues were used for [^{3}H]-5-HT and [^{3}H]-NA saturation and competition uptake experiments. The drugs tested included NA reuptake inhibitors (desipramine, atomoxetine and (*S,S*)-reboxetine), 5-HT reuptake blockers (citalopram, fluoxetine and fluvoxamine) and dual 5-HT/NA reuptake inhibitors (duloxetine and milnacipran).
Key results: In saturation experiments on synaptosomal [^{3}H]-5-HT and [^{3}H]-NA uptake, the dissociation constants did not indicate species differences although a smaller density of both SERT and NET was observed in human tissues. In competition experiments with the various drugs, marked species differences in their potencies were observed, especially at SERT. The rank order of selectivity ratios (SERT/NET) in human neocortex was as follows: citalopram ≥ duloxetine = fluvoxamine ≥ fluoxetine > milnacipran > desipramine = atomoxetine > (*S,S*)-reboxetine. Significant species differences in these ratios were observed for duloxetine, atomoxetine and desipramine.
Conclusions and implications: This study provides the first compilation of drug potency at native human neocortical SERT and NET. The significant species differences (viz., human vs. rat) in drug potency suggest that the general use of rodent data should be limited to predict clinical efficacy or profile.
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Keywords: atomoxetine; desipramine; (*S,S*)-reboxetine; citalopram; fluoxetine; fluvoxamine; duloxetine; milnacipran; noradrenaline uptake; 5-hydroxytryptamine uptake; synaptosomes; neocortex

Abbreviations: 5-HT, 5-hydroxytryptamine; DAT, dopamine transporter; NA, noradrenaline; NET, noradrenaline transporter; NRI, noradrenaline reutake inhibitor; SERT, 5-hydroxytryptamine transporter; SNRI, (dual) 5-hydroxytryptamine/noradrenaline reuptake inhibitor; SRI, 5-hydroxytryptamine reuptake inhibitor

Introduction

The transporters for noradrenaline (NA) and 5-hydroxytryptamine (5-HT), NET and SERT, respectively, represent established targets for many of the clinically relevant antidepressant drugs, commonly referred to as NA or 5-HT reuptake inhibitors (viz., NRI and SRI). The resulting increase and prolonged action of NA and 5-HT in the synaptic cleft appear to represent the basic mechanism for the clinical efficacy of these drugs (Frazer, 2001; Berton and Nestler, 2006), especially as major depression is classically linked to deficiencies in the monoamine neurotransmitters 5-HT, NA and dopamine (DA). The importance of these monoaminergic systems for the vulnerability of the central nervous system to become 'depressed' has recently been confirmed (Ruhé *et al.*,

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2007). A discrepancy exists, however, between the well-known delay (i.e. 2–3 weeks) in the clinical onset of antidepressant drug effect and the inhibition of NET and/or SERT. This discrepancy is most likely explained by adaptive changes of pre- and post-synaptic receptors, including the transition from supersensitive to normosensitive presynaptic autoreceptors, which account for the ultimate therapeutic effects of antidepressant drugs (see Göthert and Schlicker, 1997).

The human NET (hNET) was the first monoamine neurotransmitter transporter for which the amino acid sequence was identified by expression cloning (Pacholczyk *et al.*, 1991). Subsequently, the rat NET (rNET), hSERT and rat SERT (rSERT) were identified by two research groups (Blakely *et al.*, 1991; Ramamoorthy *et al.*, 1993; Bruss *et al.*, 1997). Although species variations of NET and SERT tend to be minimal [e.g. the amino acid sequence of rNET is 93% similar to that of hNET (Bruss *et al.*, 1997)], this high homology does not necessarily exclude the existence of pharmacological differences between rat and hNET and SERT respectively. For instance, in transfected cells, a higher affinity of tricyclic antidepressants has been reported for the hSERT than for the rSERT (Barker *et al.*, 1994). The significance or predictive value of results from *in vitro* studies, including those carried out in isolated cell lines, appears generally to be limited as regards the clinical situation. Despite these limitations, however, data obtained from both cell culture and animal experiments are typically extrapolated to human pharmacology. Species differences in drug potency, as an intervening or complicating variable, are often not considered in this extrapolation of results to the human patient. Therefore, the use of human native and fresh brain tissues to study the effects of NRI and SRI is important for accurate characterization of their pharmacological action in man.

In the present study, we determined and compared the potencies of various NRI, SRI and dual 5-HT and NA reuptake inhibitors (SNRI) using native NET and SERT of human and rat neocortex. The native dopamine transporter (DAT) was not studied, as preliminary experiments indicated low and variable uptake rates of DA being consistent with low levels of DAT detected in the neocortex of mammals (e.g. Wheeler *et al.*, 1993; Chalon *et al.*, 2006). To our knowledge, this is the first time the potencies of these drugs have been obtained in neocortical synaptosomes prepared from fresh specimens of human neocortex. These drugs included both classical compounds (i.e. the tricyclic antidepressant desipramine, the SRI citalopram, fluoxetine and fluvoxamine) and more recently developed agents (i.e. the SNRI duloxetine and milnacipran, the NRI atomoxetine and (*S,S*)-reboxetine). Note that some of these drugs, depending on their transporter selectivity, are used to treat fibromyalgia and other pain states, attention-deficit/hyperactivity disorder and not primarily depression.

Methods

Tissue sources

Human neocortical tissue specimens were obtained during surgical treatment of drug-resistant epilepsy or of non-epileptogenic brain tumours. Every patient signed a declaration of consent before the operation as requested by the local Ethics Committee. After pre-medication with midazolam, anaesthesia was induced with propofol and fentanyl. Cisatracurium was used for muscle relaxation. Patients received cefuroxim as an intraoperative one-time prophylactic antibiotic. The tissue was carefully removed in a gentle and nearly atraumatic manner and immediately placed in ice-cold saline to ensure viability. The neocortical samples were obtained from a total of 30 patients (14 male, 16 female; mean age: 36.8 years; youngest: 12 years; oldest: 69 years) and included frontal, temporal and parietal areas. The white matter, or macroscopically identified adherent tumorous or otherwise lesioned tissue parts, was separated (and discarded) from the grey matter that contained all six neocortical layers after preparation. The neocortical tissue was then rinsed with ice-cold physiological buffer [composition (mM): NaCl (121), KCl (1.8), $CaCl_2$ (1.3), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $NaHCO_3$ (25), glucose (10), ascorbic acid (0.06), saturated with 95% O_2/5% CO_2; pH 7.4] and processed immediately.

Wistar rats (200–300 g, n = 28; University of Freiburg, Freiburg, Germany) were maintained according to institutional policies and guidelines. All efforts were made to reduce the number of animals used and to minimize animal suffering. Animals were killed by decapitation under CO_2 anaesthesia. Each brain was quickly removed and rinsed with ice-cold buffer (composition same as above). Neocortical grey matter was isolated and synaptosomes were prepared as described below.

Preparation of synaptosomes

Freshly prepared human (0.8–1.0 g) and rat (0.5–0.8 g) neocortical tissue samples were homogenized in 10 volumes (w/v) of ice-cold sucrose (0.32 M)/HEPES (2.5 mM) buffer (pH 7.4). The following centrifugation steps were carried out: The initial homogenate was centrifuged at 1000 *g* for 10 min at 4°C (Heraeus Biofuge 28RS; Osterode, Germany). The resultant supernatant was separated and centrifuged again at 10 000 *g* for 10 min at 4°C. The supernatant from this centrifugation was discarded, and the synaptosomal pellet was resuspended in ice-cold buffer to obtain a final protein concentration of ~180 μg (human synaptosomes) or ~120 μg (rat synaptosomes) per assay tube. Protein content was determined by the method of Lowry *et al.* (1951).

[^{3}H]-noradrenaline and [^{3}H]-5-hydroxytryptamine uptake assays

Assays were carried out in physiological buffer (composition same as above) containing pargyline (5 μM) to inhibit monoamine oxidase activity and metabolism of the ^{3}H-neurotransmitter. Specific uptake was defined as total uptake minus uptake in the presence of reboxetine or (+)-oxaprotiline (10 μM) (NA uptake), and fluoxetine (10 μM) (5-HT uptake).

For a possible false labelling by [^{3}H]-5-HT or [^{3}H]-NA of dopaminergic terminals (Feuerstein *et al.*, 1986; Lupp *et al.*, 1992) to be avoided, respectively, particular attention was paid to choose inhibitors of NA uptake or of 5-HT uptake, which do not influence the DAT (e.g. Löffler *et al.*, 2006). According to this study, (+)-oxaprotiline and fluvoxamine

decreased the accumulation of [^{3}H]-dopamine in the neocortex of rats and humans. This is also true when fluoxetine instead of fluvoxamine or when reboxetine instead of (+)-oxaprotiline is used respectively (own unpublished results).

Saturation uptake experiments

Saturation characteristics of NA and 5-HT uptake were determined by diluting specific concentrations of [^{3}H]-NA and [^{3}H]-5-HT with varying amounts of the corresponding unlabelled compound to obtain final concentrations ranging from 1 nM to 10 μM ([^{3}H]-NA/NA) and from 1 nM to 3.2 μM ([^{3}H]-5-HT/5-HT) respectively. The assay was started by adding 100 μL synaptosomal suspension to 880 μL buffer followed by an 18 min incubation step at ambient temperature. Uptake was then initiated by adding 20 μL of the mixture of radiolabelled and unlabelled ligand. This second incubation period was for 20 min at 37°C to attain equilibrium. Non-specific uptake was determined for each ligand mixture concentration by using the appropriate uptake inhibitor (10 μM, see above).

Reactions were terminated by rapid filtration through glass fibre filters (GF/B, Whatman GmbH, Dassel, Germany) previously soaked in buffer containing 0.1% polyethylenimine, by using a 96-well cell harvester (Brandel M96, Gaithersburg, MD, USA). The filters were then rapidly washed with 3 mL of ice-cold buffer and transferred into scintillation vials. The washing buffer for the NA uptake experiments was pre-adjusted to pH 6.0 (with HCl), effectively reducing non-specific binding of NA to the filter. After addition of a liquid scintillation cocktail (3 mL, Ultima Gold; Packard Bioscience, Groningen, Netherlands), the filters were shaken thoroughly for 1 h. Radioactivity of the filters was determined by using a liquid scintillation analyser (Tri-Carb 2100TR; Packard Instruments, Meriden, CT, USA).

Competition uptake experiments

The assay was started by adding 100 μL synaptosomal suspension to 750 μL assay buffer and 100 μL of competing drug (concentration range: from 1 pM up to 1 mM) or assay buffer (for control). After an 18 min incubation at ambient temperature, [^{3}H]-NA or [^{3}H]-5-HT (50 μL) was added to the assay for a final concentration of 10 nM and 5 nM respectively. The second incubation period and additional details of the assay were as described above. Non-specific uptake was determined for each experiment.

Calculations and statistics

Results are given as parameter estimates and 95% confidence intervals (CI_{95}). Significant differences in the parameter estimates between humans and rats were assumed when the corresponding CI_{95} did not overlap (Gardner and Altman, 1986). Significance differences between two mean values were assessed with the Student's *t*-test. The minimal level of significance was $P \leq 0.05$ (two-tail criterion).

Note, however, that statistically significant differences may not always be pharmacologically relevant. Therefore, differences of less than 0.5 log units were not assumed to have functional implications.

Saturation and inhibition curves were generated by non-linear regression analysis (JMP 8.0, SAS Institute, Heidelberg, Germany; for applied functions, see Steffens and Feuerstein, 2004). The estimated parameters were (i) U_{max}, the asymptotic maximum of uptake [i.e. the specific uptake as pmol·mg^{-1} protein into synaptosomes that equals the number of uptake sites·mg^{-1} protein for a pure bimolecular reaction between transporter and its substrate (5-HT or NA)]; (ii) pEC_{50}, that is, the negative $\log_{10}$ of the concentration of substrate required to reach 50% of U_{max}; (iii) pIC_{50}, the negative $\log_{10}$ of the concentration of the inhibitor required to inhibit the uptake of neurotransmitter by 50%; (iv) pK_d, the negative $\log_{10}$ of the dissociation constant (K_d) between substrate and the corresponding transporter; (v) pK_i, the negative $\log_{10}$ of the inhibition constant (K_i) between the competing drug and the transporter; (vi) I_{max}, the asymptotic maximum of relative uptake inhibition (range of 0 to 1); and (vii) the slope factor *c* that estimates the existence of a bimolecular reaction between the NA or 5-HT transporter and their ligands (i.e. substrate, inhibitor) (Feuerstein and Limberger, 1999). An estimate of *c* near unity allows us to assume that the inhibitor binds either at the same uptake site as the substrate (viz., acting as a competitive antagonist), or at a distinct site that allosterically modifies the affinity of the respective substrates for the NA and 5-HT transporters. Note that increasing the number of parameters to be estimated by non-linear regression analysis of the same number of data points may increase the variations of these estimates. Thus, the variances of three parameters, for example, pIC_{50}, I_{max} and *c*, are often larger than those of only two parameters estimated simultaneously, for example, K_i and I_{max}.

As previously described (Steffens and Feuerstein, 2004), the estimate of the dissociation constant between the transporter and inhibitor was based on the difference between the descriptive IC_{50} value and K_i, mechanistically analogous to the dissociation constant. The Cheng–Prusoff equation (Cheng and Prusoff, 1973) was used to convert the IC_{50} to K_i, and this was introduced into the equation (2) of Steffens and Feuerstein to yield the following function:

$$U_{norm} = 1 - \frac{I_{max} \cdot 10^{lg[inhibitor]}}{10^{-pK_i} + 10^{lg[substrate] - pK_i + pK_d} + 10^{lg[inhibitor]}} \qquad (1)$$

Materials

The radiolabelled substances were *l*-[*ring*-2,5,6-^{3}H]-noradrenaline (1.92 TBq·mmol^{-1}; DuPont, Dreieich, Germany) and [1,2-^{3}H(N)]-5-hydroxytryptamine (1.11 TBq·mmol^{-1}; Perkin-Elmer, Wiesbaden, Germany). Other substances included citalopram hydrobromide, desipramine hydrochloride, fluoxetine hydrochloride, fluvoxamine maleate, 5-hydroxytryptamine hydrochloride, milnacipran hydrochloride, *l*-noradrenaline hydrochloride, and pargyline hydrochloride (Sigma-Aldrich, Taufkirchen, Germany); (*S,S*)-reboxetine methanesulphonate (Pfizer, Ann Arbor, MI, USA); (+)-oxaprotiline hydrochloride (Novartis, Basel, Switzerland); and reboxetine mesylate (Tocris, Bristol, UK). Atomoxetine hydrochloride and duloxetine hydrochloride were isolated from capsules/tablets obtained commercially. The substances

Table 1 Parameters of saturation experiments on [^{3}H]-5-hydroxytryptamine and [^{3}H]-noradrenaline uptake into human and rat neocortical synaptosomes

	pEC_{50}	U_{max} (pmol·mg^{-1} protein)	c
[^{3}H]-5-HT			
Human	7.32 [6.72, 7.74]	16.27 [10.27, 29.60]***	0.99 [0.80, 1.23]
Rat[§]	7.56 [7.45, 7.66]	39.98 [36.66, 43.72]	0.91 [0.85, 0.97]
[^{3}H]-NA			
Human	6.46 [5.67, 6.85]	4.68 [2.73, 12.52]***	1.17 [0.88, 1.56]**
Rat	6.64 [6.35, 6.87]	35.85 [29.72, 44.20]	0.72 [0.66, 0.79]

Values given are estimates [CI_{95}] ($n \geq 3$ independent experiments, at least six concentrations of drug/experiment, each concentration in six replicates).
A significant difference from the corresponding value for rat neocortex is indicated by asterisks (**$P \leq 0.01$, ***$P \leq 0.001$).
[§]Data are from Steffens and Feuerstein (2004).

were initially dissolved in water (10 mM stock) and then further diluted with assay buffer.

Results

Saturation uptake experiments

The saturation uptake experiments determined the kinetic parameters of SERT and NET in human and rat neocortical synaptosomes respectively. The pEC_{50}, U_{max} and slope factor *c* values for 5-HT and NA uptake into synaptosomes from both species are given in Table 1. Specific uptake of [^{3}H]-5-HT and [^{3}H]-NA for either human or rat synaptosomes yielded pEC_{50} values in the high nanomolar range. Because these pEC_{50} estimates are assumed to represent pK_d values due to *c* near 1 (see 'Discussion'), species differences were not evident in the affinity of [^{3}H]-5-HT and [^{3}H]-NA to their respective transporters.

In contrast, the number of uptake sites (U_{max}) for [^{3}H]-5-HT and [^{3}H]-NA was significantly lower in human (59 and 87% respectively) than in rat neocortical synaptosomes. Because three of the slope factors *c* (i.e. Hill coefficients) were near unity, single NA- and 5-HT-uptake sites were assumed for the corresponding bimolecular substrate–transporter interactions. The slope factor *c* for specific NA uptake in the rat was, however, below unity.

Effect of various reuptake inhibitor drugs on [^{3}H]-5-hydroxytryptamine uptake

The inhibitory effects of the tested drugs on [^{3}H]-5-HT uptake into human and rat neocortical synaptosomes, respectively, are presented in Table 2A.

The inhibition by each compound was concentration dependent and is exemplified by the concentration–inhibition curve for atomoxetine in both species (Figure 1). The pIC_{50}, I_{max} and *c* values are listed in Table 2A. All pIC_{50} values, with the exception of fluoxetine, were significantly different between human and rat neocortical synaptosomes (see also Figure 1). The pIC_{50}-values for desipramine and atomoxetine were relevantly higher (i.e. by more than 0.5 log units) in human than in rat neocortex, contrasting with the pIC_{50} value for (*S*,*S*)-reboxetine that was relevantly higher in rat. All I_{max} values approximated to unity with no evidence of species differences. The values of the slope factor *c* differed from unity for (*S*,*S*)-reboxetine in rat and for duloxetine in both species. The slope factors *c* varied markedly around unity for citalopram, fluoxetine and fluvoxamine in rat and for atomoxetine in both rat and human.

On the basis of an assumed $c = 1$, a pK_d for [^{3}H]-5-HT of 7.32 (human) and 7.56 (rat, see Table 1) and a concentration of [^{3}H]-5-HT of 5 nM in the uptake inhibition experiments, the pK_i values for the different reuptake inhibitors were calculated (see 'Methods') and presented in Table 3.

All drugs, with the exception of fluoxetine, gave significantly different pK_i values for [^{3}H]-5-HT uptake between the human and rat. Relevant potency differences, however, were only seen for desipramine and atomoxetine. The SNRI duloxetine was the most potent compound, having K_i values less than 1 nM in both human and rat neocortical synaptosomes (Table 3).

The rank order of inhibitory potency of the drugs for hSERT was as follows: duloxetine > citalopram > fluvoxamine ≥ fluoxetine ≥ milnacipran ≥ atomoxetine > desipramine > (*S*,*S*)-reboxetine. The rank potency order for rSERT was similar: duloxetine > citalopram > fluvoxamine > fluoxetine ≥ milnacipran > atomoxetine > desipramine > (*S*,*S*)-reboxetine.

Effect of various reuptake inhibitors on [^{3}H]-noradrenaline uptake

The inhibitory effects of the tested drugs on [^{3}H]-NA uptake into human and rat neocortical synaptosomes, respectively, are listed in Table 2B. All drugs produced concentration-dependent inhibitory effects on [^{3}H]-NA uptake, again similar to atomoxetine (Figure 1).

The pIC_{50}, I_{max} and *c* values are given in Table 2B. Fluvoxamine and (*S*,*S*)-reboxetine were the only drugs with pIC_{50} estimates differing by more than 0.5 log units between species. Some of the maxima of relative inhibition (I_{max}) of [^{3}H]-NA uptake into human synaptosomes, as indicated for citalopram, fluoxetine and duloxetine, varied markedly around unity. Similar high variations around unity were also noted for the slope factor *c*, including desipramine and fluoxetine (both human and rat), and citalopram, fluvoxamine, atomoxetine, (*S*,*S*)-reboxetine, duloxetine and milnacipran (human). Despite the high variability of *c* for fluvoxamine, atomoxetine and (*S*,*S*)-reboxetine in the rat, these slope factors were different from unity.

The apparent pK_i values (Table 3) for all reuptake inhibitor drugs were estimated, assuming $c = 1$, pK_d for [^{3}H]-NA/NA of 6.46 (human neocortex) or 6.64 (rat neocortex, Table 1) and the concentration of [^{3}H]-NA (10 nM) in the uptake inhibi-

Table 2 (A) Parameters of inhibition experiments with various reuptake inhibitor drugs on [^{3}H]-5-hydroxytryptamine uptake into human and rat neocortical synaptosomes. (B) Parameters of inhibition experiments with various reuptake inhibitor drugs on [^{3}H]-noradrenaline uptake into human and rat neocortical synaptosomes

	pIC_{50}	I_{max}	c
A			
Citalopram			
Human	8.44 (8.32, 8.56)***	0.88 (0.85, 0.92)	1.04 (0.83, 1.35)
Rat	8.78 (8.68, 8.88)	0.97 (0.93, 1.01)	1.21 (0.96, 1.57)
Fluvoxamine			
Human§	7.96 (7.74, 8.15)**	0.95 (0.89, 1.03)	0.84 (0.60, 1.24)
Rat§	8.32 (8.21, 8.43)	1.00 (0.97, 1.04)	0.96 (0.798, 1.18)
Duloxetine			
Human	9.26 (9.09, 9.41)**	0.95 (0.91, 1.00)	0.43 (0.38, 0.50)
Rat	8.81 (8.52, 9.04)	1.05 (0.97, 1.15)	0.44 (0.36, 0.54)
Fluoxetine			
Human	7.85 (6.57, 8.30)	1.09 (0.91, 1.70)	0.95 (0.28, –)
Rat	7.48 (7.38, 7.58)	1.00 (0.95, 1.08)	1.01 (0.95, 1.08)
Milnacipran			
Human	7.80 (7.66, 7.93)***	0.93 (0.88, 0.99)	0.95 (0.77, 1.20)
Rat	7.39 (7.25, 7.53)	1.01 (0.95, 1.07)	0.79 (0.64, 1.00)
Atomoxetine			
Human	7.67 (7.51, 7.81)***	0.93 (0.88, 1.00)	1.00 (0.74, 1.49)
Rat	6.77 (6.65, 6.89)	1.00 (0.94, 1.04)	1.13 (0.88, 1.49)
Desipramine			
Human	7.16 (6.93, 7.34)***	0.96 (0.88, 1.06)	0.80 (0.58, 1.12)
Rat	5.97 (5.67, 6.11)	1.02 (0.91, 1.27)	1.19 (–, –)
(*S,S*)-Reboxetine			
Human	4.55 (4.18, 4.79)**	1.11 (0.97, 1.38)	0.88 (0.66, 1.24)
Rat	5.08 (4.85, 5.21)	0.89 (0.77, 1.12)	1.35 (1.01, 1.77)
B			
Citalopram			
Human	5.03 (2.57, 5.73)	0.98 (0.68, 3.28)	0.66 (0.34, 1.35)
Rat	5.35 (5.21, 5.46)	1.01 (0.94, 1.10)	0.92 (0.75, 1.14)
Fluvoxamine			
Human	5.62 (5.29, 5.88)***	1.06 (0.94, 1.21)	1.24 (0.75, 2.48)
Rat	6.17 (6.07, 6.29)	0.80 (0.73, 0.87)	1.97 (1.42, 2.79)
Duloxetine			
Human	6.89 (5.44, 7.43)	1.04 (0.80, 1.84)	0.71 (0.34, 1.55)
Rat	7.40 (7.26, 7.53)	0.91 (0.86, 0.98)	0.88 (0.70, 1.11)
Fluoxetine			
Human	5.94 (4.45, 7.14)	0.90 (0.43, 3.09)	0.66 (–, –)
Rat	6.45 (6.07, 6.65)	0.95 (0.83, 1.18)	0.86 (0.57, 1.34)
Milnacipram			
Human	7.81 (7.31, 8.25)	0.90 (0.71, 1.12)	1.07 (0.58, 3.21)
Rat	7.44 (7.31, 7.55)	0.82 (0.78, 0.88)	0.95 (0.77, 1.19)
Atomoxetine			
Human	9.51 (9.17, 9.83)	0.92 (0.82, 1.03)	0.83 (0.54, 1.39)
Rat	9.58 (9.41, 9.74)	0.90 (0.87, 0.94)	0.55 (0.44, 0.71)
Desipramine			
Human	9.13 (8.83, 9.40)	0.88 (0.77, 1.01)	0.85 (0.58, 1.40)
Rat	9.33 (9.11, 9.56)	0.95 (0.89, 1.02)	1.04 (0.65, 1.77)
(*S,S*)-Reboxetine			
Human	8.37 (7.89, 8.76)**	0.87 (0.73, 1.05)	0.96 (0.54, 2.04)
Rat	8.98 (8.93, 9.04)	0.75 (0.73, 0.77)	1.83 (1.53, 2.27)

Values given are estimates [CI_{95}] ($n \geq 2$ independent experiments, at least six concentrations of drug/experiment, each concentration in six replicates).
A significant difference from the corresponding value for rat neocortex is indicated by asterisks (**$P \leq 0.01$, ***$P \leq 0.001$).
§Data from Lieb *et al.* (2005).

tion experiments (Table 1). Significant and relevant differences in pK_i values for [^{3}H]-NA uptake inhibition between human and rat were only observed for fluvoxamine and (*S,S*)-reboxetine. The NRI atomoxetine was the most potent compound (subnanomolar range) in inhibiting [^{3}H]-NA uptake in both species, whereas citalopram was the least potent drug (Table 3).

The rank order of inhibitory potency of the drugs for hNET was as follows: atomoxetine ≥ desipramine > (*S,S*)-reboxetine ≥ milnacipran ≥ duloxetine ≥ fluoxetine ≥ fluvoxamine ≥ citalopram. The rank potency order for rNET was similar: atomoxetine ≥ desipramine > (*S,S*)-reboxetine > milnacipran ≥ duloxetine > fluoxetine > fluvoxamine > citalopram.

Selectivity profile of reuptake inhibitor drugs

Selectivity ratios [$pK_{i(5\text{-}HT)}$ minus $pK_{i(NA)}$] of the various reuptake inhibitor drugs, greater than or less than unity (i.e. significant differences in pK_i estimates; compare Harms, 1983) across transporter and species, are depicted in Figure 2.

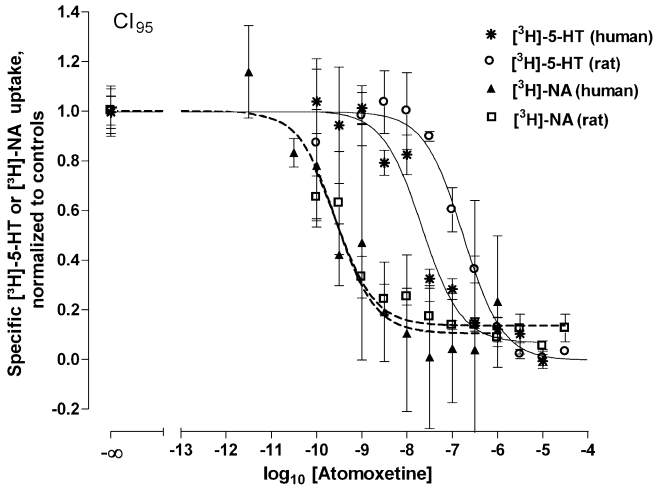

Figure 1 Inhibition of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) and [^{3}H]-noradrenaline ([^{3}H]-NA) uptake into human and rat neocortical synaptosomes by atomoxetine. Synaptosomes were incubated (20 min, 37°C) with 5 nM [^{3}H]-5-HT or 10 nM [^{3}H]-NA in the presence of various concentrations of atomoxetine. Nonspecific uptake was determined by using 10 μM fluoxetine (5-HT) or 10 μM reboxetine (NA). Results are given as means with Cl_{95} ($n \geq 6$ determinations) with fitted curves according to function (2) of Steffens and Feuerstein (2004).

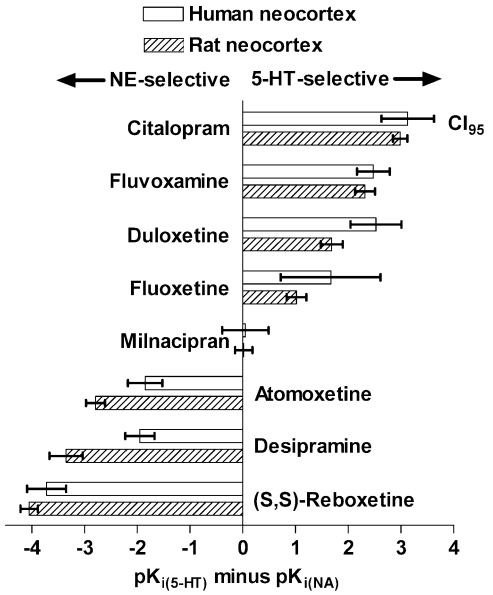

Figure 2 Selectivity profile of several reuptake inhibitor drugs for [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) and [^{3}H]-noradrenaline ([^{3}H]-NA) uptake into human and rat synaptosomes. The selectivity index was defined as $pK_{i\ (5\text{-}HT)}$ minus $pK_{i\ (NA)}$, corresponding to $K_{i(5\text{-}HT)}/K_{i(NA)}$.

Table 3 Estimates of pK_i for various drugs inhibiting [^{3}H]-5-hydroxytryptamine and [^{3}H]-noradrenaline uptake into human and rat neocortical synaptosomes

	$pK_{i\ (5\text{-}HT)}$	$pK_{i\ (NE)}$
Citalopram		
Human	8.48 (8.37, 8.60)***	5.35 (4.88, 5.85)
Rat	8.83 (8.73, 8.93)	5.39 (5.30, 5.49)
Fluvoxamine		
Human	8.04 (7.87, 8.21)***	5.56 (5.30, 5.82)***
Rat	8.39 (8.29, 8.50)	6.07 (5.91, 6.23)
Duloxetine		
Human	9.55 (9.41, 9.70)***	7.02 (6.55, 7.47)
Rat	9.14 (8.97, 9.32)	7.45 (7.34, 7.56)
Fluoxetine		
Human	7.90 (7.50, 8.33)	6.23 (5.46, 7.15)
Rat	7.54 (7.45, 7.64)	6.52 (6.36, 6.68)
Milnacipran		
Human	7.85 (7.73, 7.97)***	7.80 (7.38, 8.23)
Rat	7.49 (7.37, 7.62)	7.47 (7.36, 7.58)
Atomoxetine		
Human	7.71 (7.58, 7.85)***	9.56 (9.26, 9.86)
Rat	6.83 (6.71, 6.96)	9.62 (9.49, 9.75)
Desipramine		
Human	7.24 (7.10, 7.40)***	9.19 (8.96, 9.43)
Rat	6.00 (5.70, 6.14)	9.35 (9.13, 9.58)
(*S,S*)-Reboxetine		
Human	4.68 (4.54, 4.81)**	8.40 (8.06, 8.75)***
Rat	4.94 (4.79, 5.08)	8.99 (8.92, 9.08)

Values given are estimates [Cl_{95}] ($n \geq 2$ independent experiments, at least 6 concentrations of drug/experiment, each concentration in six replicates).
A significant difference from the corresponding value for rat neocortex is indicated by asterisks (**$P \leq 0.01$, ***$P \leq 0.001$).

The rank order of selectivity varied primarily as a function of the ^{3}H-monoamine substrate rather than species. For hSERT the order was citalopram ≥ duloxetine = fluvoxamine ≥ fluoxetine, being very similar to that for rSERT: citalopram > fluvoxamine > duloxetine > fluoxetine. For hNET, the order was (*S,S*)-reboxetine > desipramine = atomoxetine, again very similar to that for rNET: (*S,S*)-reboxetine > desipramine > atomoxetine. In both species, desipramine, (*S,S*)-reboxetine and atomoxetine exhibited markedly higher potency as inhibitors of [^{3}H]-NA than of [^{3}H]-5-HT uptake, whereas all the other compounds (except milnacipran) were selective for [^{3}H]-5-HT uptake. (*S,S*)-Reboxetine was clearly the most selective NRI in both species, and citalopram was the most selective SRI. Milnacipran was non-selective on SERT and NET and can be classified as a dual-action inhibitor. Significant species differences for the reuptake inhibitor drugs were most evident for desipramine, atomoxetine and duloxetine (Figure 2).

Discussion

In the present study, possible pharmacological differences between human and rat neocortical SERT and NET were assessed by measuring the selectivity and potency of several reuptake inhibitor drugs. This is the first study, to our knowledge, that directly compares the effects of various NRI, SRI and SNRI on [^{3}H]-5-HT and [^{3}H]-NA uptake in synaptosomes prepared from freshly obtained human and rat neocortical tissues.

Because differences in assay conditions between laboratories (e.g. radiolabelled substrate) should only minimally affect the comparison of parameter estimates in different studies (Cheng and Prusoff, 1973) and because mechanistically elucidated parameters are to be preferred over descriptive parameters (Feuerstein and Limberger, 1999; Feuerstein and Sauermann, 2005), K_i and K_d values were calculated rather than IC_{50} and EC_{50} values. The calculation of these dissociation constants requires the assumption of bimolecular reac-

tions between the transporter and its ligand or substrate (Feuerstein and Limberger, 1999). A slope factor *c* around unity suggests that such a bimolecular reaction occurs, which allows the assumption that $EC_{50} = pK_d$. As shown in Table 1, however, the *c* value for specific [^{3}H]-NA uptake into rat synaptosomes was below unity and, thus, precludes assuming bimolecularity. This means that the pEC_{50} estimate may not exactly reflect a pK_d in the case of rat neocortex and that the interpretation of U_{max} as number of uptake sites·mg^{-1} protein must be considered with caution. The difficulty of comparing, for instance, IC_{50} estimates between laboratories can be illustrated for fluoxetine and desipramine at SERT and NET. Langer *et al*. (1980) published rather different pIC_{50}s from our data: 6.25 (SERT, rat hypothalamic slices) versus 7.48 (SERT, rat neocortical synaptosomes); 5.32 (SERT, rat hypothalamic slices) versus 5.97 (SERT, rat neocortical synaptosomes); 5.45 (NET, rat hypothalamic slices) versus 6.45 (NET, rat neocortical synaptosomes); 7.70 (NET, rat hypothalamic slices) versus of 9.33 (NET, rat neocortical synaptosomes). These values differ significantly; the exact reasons for such discrepancies are unknown although assay conditions, source of transporter (i.e. slice vs. synaptosomes) and shapes of concentration–inhibition curves can all be considered essential factors.

The pK_d values, for the substrates 5-HT and NA and their respective transporters SERT and NET, did not significantly differ between human and rat neocortical synaptosomes. As K_d reflects the degree of spatial fit between a drug and the three-dimensional receptor surface, the similarity of the pK_d values for 5-HT and NA transport into neocortical synaptosomes suggests a highly homologous substrate binding site in neocortical hSERT and rSERT, and hNET and rNET respectively. Indeed, the amino acid sequence of rNET is 93% similar to that of hNET (Bruss *et al*., 1997), and rSERT resembles hSERT by 92% (Ramamoorthy *et al*., 1993).

In comparison with the present data for the SERT K_d, [i.e. 48 nM (human) and 28 nM (rat)], Mann and Hrdina (1992) reported a higher K_d (210 nM; incubation time of 2 min) for [^{3}H]-5-HT uptake into rat hypothalamic synaptosomes. Wood (1987), however, reported a K_d of 72 nM with rat neocortical synaptosomes (incubation time of 4 or 6 min), approximating the current results (incubation time of 20 min). Because the affinity of the accumulation of 5-HT increases with the incubation time over the first few minutes (see Wood, 1987), the short incubation period of Mann and Hrdina may explain their higher 1/affinity estimate. A recent estimate of the pK_m on [^{3}H]-NA uptake into rat neocortical synaptosomes was 6.66 (Jeannotte and Sidhu, 2008), nearly identical to our rat pK_d value of 6.64.

In contrast to the interspecies similarity of our pK_d values, the number of uptake sites of·mg^{-1} protein (U_{max}) for both SERT and NET was significantly lower in human than in rat neocortical synaptosomes, suggesting that the density of both transporters in human tissue is considerably less than in rat tissue. The above-mentioned papers (Wood, 1987; Mann and Hrdina, 1992; Jeannotte and Sidhu, 2008) do not provide V_{max} or U_{max} estimates obtained at equilibrium conditions for comparison with our U_{max} values. This is because the V_{max} values of these studies reflect incubation times of $\leq$6 min with the respective ^{3}H-monoamine (as compared with 20 min in the present investigation).

As regards the inhibitory effects of the reuptake inhibitor drugs on hSERT, rSERT, hNET and rNET, all substances reduced the uptake of both [^{3}H]-5-HT and [^{3}H]-NA into human and rat neocortical synaptosomes in a concentration-dependent manner (refer to Figure 1). The IC_{50} values, or the corresponding K_i values, of these drugs using rat neocortex were generally consistent with literature values (Hyttel, 1994; Sanchez and Hyttel, 1999; Hajos *et al*., 2004; Zhou, 2004; Stahl *et al*., 2005). The present study, in contrast to some of these cited, gave attention to the shape of the concentration–response curves and the estimates of the slope factor *c*. If the slope factor resembles unity, then there is a high probability of a bimolecular reaction between the SERT or NET and the inhibitor of these transporters (Feuerstein and Limberger, 1999; Feuerstein and Sauermann, 2005). The probability of a pure bimolecular reaction is also dependent on the range of the CI_{95} encompassing unity (see Table 2). Only a narrow CI_{95} of *c* that includes unity is compatible with the law of mass action describing a bimolecular reaction (see Feuerstein and Limberger, 1999).

Among the SRI tested in the present study, citalopram was the most potent, and fluoxetine was the least potent inhibitor of [^{3}H]-5-HT uptake. Our data for SRIs in rat neocortical synaptosomes are consistent with literature values for IC_{50} (Frazer, 2001) and affinity (Richelson and Pfenning, 1984; Sanchez and Hyttel, 1999). The slightly higher affinity of citalopram at rSERT, compared with hSERT, agrees with previous results from binding assays (Plenge and Mellerup, 1991). Fluvoxamine also had a significantly lower affinity to hNET, an observation further supported by binding studies that compared affinities of rat and human neocortical NET with NET-transfected cells (Owens *et al*., 1997). All tested SRIs were selective to varying degrees at inhibiting [^{3}H]-5-HT uptake. The rank order of this SERT selectivity did not differ between human and rat neocortex. Citalopram was the most selective SRI, and fluoxetine was the least, entirely consistent with results from animal studies (Richelson and Pfenning, 1984; Hyttel, 1994; Frazer, 2001).

Duloxetine and milnacipran block the reuptake of both [^{3}H]-5-HT and ^{3}H]-NA with differing selectivity (Stahl *et al*., 2005). In the present study, milnacipran blocked both [^{3}H]-5-HT and [^{3}H]-NA uptake with similar potency and, therefore, without selectivity. Duloxetine, in contrast, was both more potent and SERT selective. Our data on the potency of these drugs in both species are in agreement with binding and uptake studies (Wong *et al*., 1993; Beique *et al*., 1998; Bymaster *et al*., 2001; Stahl *et al*., 2005). The equivalent potency of milnacipran at the hSERT and hNET is consistent with previous results from binding and uptake studies on cells transfected with these transporters (Vaishnavi *et al*., 2004). A preferential action of duloxetine at SERT has also been observed functionally *in vivo* by using an electrophysiological paradigm and in *ex vivo* uptake studies (Kasamo *et al*., 1996).

As expected, desipramine was more potent at inhibiting [^{3}H]-NA than [^{3}H]-5-HT uptake, corroborating results of other researchers (Richelson and Pfenning, 1984; Hyttel, 1994; Sanchez and Hyttel, 1999; Frazer, 2001). Although desipramine was more potent on [^{3}H]-NA uptake, its pK_i for [^{3}H]-5-HT uptake differed substantially between human and rat with notably higher affinity for hSERT. The higher potency

of desipramine at the hSERT (K_i = 58 nM) is consistent with previous findings using hSERT-transfected cells (Barker *et al.*, 1994; Barker and Blakely, 1996; Owens *et al.*, 1997). This observation suggests that only a minor portion of desipramine's clinical efficacy reflects 5-HT uptake inhibition. Surprisingly, however, although desipramine was NET-selective in both species, the 90-fold higher selectivity for hNET over hSERT contrasted sharply to the >2200-fold higher NET selectivity in rats (Figure 2). This higher selectivity of desipramine for the rNET also agrees with results from binding studies comparing neocortical rNET and cells transfected with hNET (Owens *et al.*, 1997). In contrast, Harms (1983) did not find a significant difference in selectivity for desipramine between human and rat brain slices. The discrepancy between the present results and those of Harms probably relates to the use of slices in the latter study rather than synaptosomes, and the condition of the human tissue (*post-mortem* vs. fresh). Thus, our results emphasize an important species difference in the selectivity of desipramine.

Some NRI drugs (e.g. reboxetine, atomoxetine) have been developed to treat a variety of psychiatric disorders including depression and attention-deficit/hyperactivity disorder (Zhou, 2004). In the present study, the NRI atomoxetine displayed the highest NET pK_i (Table 3), whereas the selectivity to inhibit [^{3}H]-NA uptake was considerably higher in rat neocortical synaptosomes (Figure 2). In uptake studies in cells transfected with cloned hNET and hSERT, atomoxetine exhibited a higher pK_i at the NET (Zhou, 2004). Note also that atomoxetine, although binding with high affinity to NET, also inhibits [^{3}H]-5-HT uptake with nanomolar potency and there are significant, relevant differences between human and rat neocortex (19 nM vs. 148 nM, respectively; Table 3). The selectivity of atomoxetine for NET and its slightly lower affinity for the 5-HT uptake site was also confirmed in binding studies (Gehlert *et al.*, 1995).

The NRI reboxetine has also been shown to exhibit a high affinity for the hNET and rNET (Wong *et al.*, 2000; Hajos *et al.*, 2004). The (*S,S*)-enantiomer is even more potent and selective at the NET than the racemate (i.e. (*R,R*)- and (*S,S*)-enantiomers of reboxetine; Zhou (2004). In the present study, the pK_i value of (*S,S*)-reboxetine (8.99) for the rat rNET (Table 3) was nearly identical to that observed in binding studies (9.0; Hajos *et al.*, 2004) and higher than that reported for the racemate tested in rat hypothalamic synaptosomes (8.1; Hajos *et al.*, 2004). The pK_i of (*S,S*)-reboxetine (8.40) observed in human neocortex in the present investigation (Table 3) is, however, higher than that reported for reboxetine in Madin-Darby canine kidney cells expressing hNET (7.96; Wong *et al.*, 2000; Hajos *et al.*, 2004). In contrast, (*S,S*)-reboxetine was found to be an extremely weak inhibitor of [^{3}H]-5-HT uptake in both species (Table 3). The selectivity of (*S,S*)-reboxetine for hNET and rNET was 5248-fold and 11 220-fold, being very similar to previous findings for rNET (Hajos *et al.*, 2004). All of these results support the contention (Hajos *et al.*, 2004; Zhou, 2004) that (*S,S*)-reboxetine is one of the most potent and selective NRI known or available. Finally, *(S,S)*-reboxetine also exhibits a relevant species difference as indicated by its higher pK_i value for [^{3}H]-NA uptake in the rat neocortex (Table 3).

In conclusion, the effects of various reuptake inhibitor drugs on SERT and NET function have been characterized for the first time by using native, fresh human neocortical tissue. The targets of these drugs are considered to be SERT and NET localized to neocortical 5-hydroxytrytaminergic and noradrenergic axon terminals. As shown here, however, relevant species differences can exist for these drugs even though the endogenous substrates, 5-HT and NA, are bound with the same affinity by SERT and NET of both species. Therefore, pharmacological inhibition of [^{3}H]-NA and [^{3}H]-5-HT uptake in rat neocortical synaptosomes cannot always be extrapolated to similar properties in human brain. Moreover, the present findings may help to predict more precisely the profile of antidepressants in their clinical applications.

However, the present paper does not deal with DA transport blockers, and we must not forget that a decrease in dopaminergic transmission may also be one of the neurochemical alterations in depression (for a further discussion of dopamine in the context of antidepressant therapy, see Feuerstein, 2007).

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