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Molecular Brain Research 71 (1999) 120–126

MOLECULAR BRAIN RESEARCH

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Research report

Molecular cloning, expression and characterization of a bovine serotonin transporter [1]

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Accepted 11 May 1999

Abstract

The serotonin transporter (SERT) is a member of a highly homologous family of sodium/chloride dependent neurotransmitter transporters responsible for reuptake of biogenic amines from the extracellular fluid. SERT constitutes the pharmacological target of several clinically important antidepressants. Here we report the molecular cloning of SERT from the bovine species. Translation of the nucleotide sequence revealed 44 amino acid differences compared to human SERT. When transiently expressed in HeLa cells and compared with rat and human SERTs the K_m value for uptake was increased 2-fold. V_{max} and B_{max} were both increased about 4-fold indicating the turnover number is conserved. The pharmacological profile revealed a decreased sensitivity towards imipramine, desipramine, citalopram, fluoxetine and paroxetine compared with human SERT, while the sensitivity towards 3,4-methylenedioxymethamphetamine (MDMA) was mainly unchanged. RT-PCR amplification of RNA from different tissues demonstrated expression of SERT in placenta, brain stem, bone marrow, kidney, lung, heart, adrenal gland, liver, parathyroid gland, thyroid gland, small intestine and pancreas. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Serotonin transporters; Uptake parameters; Pharmacological profile; SSRI; TCA; RT-PCR

1. Introduction

The biogenic amine serotonin (5-hydroxytryptamine) is a neurotransmitter in both the central, as well as in the peripheral nervous system. Following transmitter release, neural transmission is regulated and terminated by active reuptake of the neurotransmitter from the extracellular fluid. The uptake process is accomplished by SERT. The transmitter is then either degraded by monoamine oxidases or repackaged into vesicles by the proton dependent vesicular monoamine transporter. SERT is member of a large superfamily of sodium/chloride dependent transporters with substrates including biogenic amines, glycine, taurine, proline, creatine, betaine and γ-aminobutyric acid [1,18].

The sodium/chloride dependent transporters consist of approximately 600 amino acid residues and they share a structural motif of 12 putative transmembrane domains (TMDs) [5]. The transporters possess glycosylation consensus sites in the large extracellular loop between TMD3 and TMD4 and they also have several potential intracellular phosphorylation sites in common. Recently SERT was shown to be phosphorylated following protein kinase C activation [24].

Serotonin is cotransported inwardly in a protonated form with one Na^+ ion and one Cl^- ion and one K^+ is transported outwards, as the transporter returns to its initial state. Recently, however, transport of serotonin was reported to be associated with a net inward current as measured by electrophysiological techniques [16]. Most likely, this current arises from uncoupled transport of additional Na^+ ions.

SERT has received much attention as it represents the pharmacological target of tricyclic antidepressants and serotonin selective reuptake inhibitors (SSRIs), which are widely used in the treatment of depression, anxiety disorders, obsessive-compulsive disorder and eating disorders [10]. Furthermore, SERT is the target of several drugs of abuse such as cocaine and amphetamines.

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[1] The sequence reported in this paper has been deposited in the GenBank data base (accession number AF119122).

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PII: S0169-328X(99)00178-3

The human gene contains two different polymorphic repeat regions which in some families have been shown to be associated with depression and anxiety, respectively [14,19], suggesting that SERT may be involved in the pathophysiology of these disorders.

SERT cDNA was first cloned from rat [3,12] and has so far been cloned from human [15,23], mouse [4], drosophila [8,9], guinea pig [6] and sheep [21]. They all share high homology, with drosophila as the most distant relative.

Serotonin reuptake has been documented in various bovine tissues [13,28]. It is a process characterized by a saturable high affinity uptake and dependent on both sodium and chloride ions. Uptake by the bovine transporter is imipramine sensitive [13] and in cow has been found in platelets, in endothelial cells and in retina [13,20,26,28].

We here report the molecular cloning of the bovine SERT (bSERT) by RT-PCR amplification of bovine placental mRNA. The expression pattern of SERT was examined in several different bovine tissues. The cloned cDNA was sequenced and characterized by transfection and functional expression in HeLa cells. Uptake parameters, K_m and V_{max}, were increased and the pharmacological profile was different when compared with human SERT (hSERT) and rat SERT (rSERT).

2. Materials and methods

2.1. Materials

DMEM media was obtained from Life Technologies. Cell culture flasks and 96-well plates were from NUNC. Fetal bovine serum was from Life Technologies. [^{3}H]-serotonin (21.7 Ci/mmol), [^{3}H]-norepinephrine (10.2 Ci/mmol) and [^{125}I]-RTI-55(2200 Ci/mmol) were from NEN-DuPont and [^{14}C] – dopamine (57 mCi/mmol) from Amersham. Citalopram was a gift from H. Lundbeck, paroxetin from SmithKline Beecham, fluoxetin from EliLilly and (±)-3,4-methylenedioxymethamphetamine (MDMA), imipramine and desipramine were purchased from Sigma.

2.2. Cloning

Total RNA was isolated from bovine placenta using an acid guanidinium thiocyanate method by Chomczynski and Sacchi [7]. Single stranded cDNA was synthezised from 5 μg of total RNA using Superscript II reverse transcriptase (BRL) and a universal oligo-dT-anchor primer (GACCACGCGTATCGATGTCGACT$_{16}$V) at 42°C for 60 min. Complementary RNA was removed with RNase H.

PCR was performed using the following primers, which correspond to regions that are identical in mammalian SERTs and located at amino acid positions 42–47 and 216–221, respectively; SERT5′(CAAATATCCAATGGGTACTC) and SERT3′(GTCCAGGTGATGTTGTCCT). Thirty-five cycles of PCR amplification were carried out as follows: 1 min at 94°C, 1 min at 60°C and 1 min at 72°. The generated 550 bp fragment was cloned into AT cloning vector pGEM-T-easy (Promega) and sequenced using an automated sequencer ABI310 (Perkin Elmer).

From this new sequence, a bovine specific primer bSERT2 (TCACGGAGCAGCTGCCCTGGACC) was constructed and used with the universal anchorprimer (GACCACGCGTATCGATGTCGAC) to amplify the 3′ region. Thirty-five cycles of PCR amplification were carried out as follows: 1 min at 94°C, 1 min at 65°C and 4 min at 72°C. PCR amplification generated a 2 kb fragment.

Full-length cDNA was constructed using degenerate primer bSERT-5′ (TCAYYTMCTARCCAGCAGGATG) and the bovine specific primer bSERT-3′ (AGCACACAGGTTACAGGGTTCT). Thirty-five cycles of PCR amplification were carried out as follows: 1 min at 94°C, 1 min at 60°C and 4 min at 72°C. In all PCR reactions the proofreading enzyme Klentaq polymerase (Clontech) was used.

The full-length cDNA was amplified and cloned independently several times to identify PCR artifacts. pGEM-T-easy was used as cloning vehicle.

All constructions were sequenced by the Dye Terminator Cycle Sequencing method on an ABI310 automated sequencer.

An *Eco*RI/*Nde*I fragment containing the entire bSERT coding sequence and 440 bp of the 3′ untranslated region (UTR) was further subcloned into the *Eco*RI/*Eco*RV restriction sites of the eucaryotic expression vector pcDNA3 (In Vitrogen). hSERT cDNA was cloned with RT-PCR from human placenta [17], primer SHTT14 (CAAACTCATTCACTCGGAGGGGA) was used for 1. strand synthesis and primers 5HTT13 (CAGGCGTGCCTCCTCAGAACT) and SHTT15 (ATCCTTGGCAGATGGACATCAGTG) for PCR amplification. The coding sequence was subsequently subcloned into the *Xho*I/*Xba*I sites of pcDNA3. A pcDNA3 clone with rSERT was kindly donated by Beth J. Hoffman, NIMH. This clone contained 232 bps of the 5′ UTR, the coding sequence and 558 bps of the 3′ UTR cloned into the *Xba*I site of pcDNA3 [12].

2.3. RT-PCR

Total RNA was isolated from bovine tissue by the method of Chomczynski and Sacchi [7]. Single-stranded cDNA was synthesised from 5 μg of total RNA using MMLV reverse transcriptase (Promega) and the genespecific primer bF (GCATCACAGAGTCTAACGTG) at 37°C for 60 min. The cDNA was amplified with primers b2 (TCACGGAGCAGCTGCCCTGGACC) and bI (TGCTGTCCAAGCCCAGGG) and Taq polymerase (Promega) (1 min at 94°C, 1 min at 63°C and 1 min at 72°C for 35 cycles).

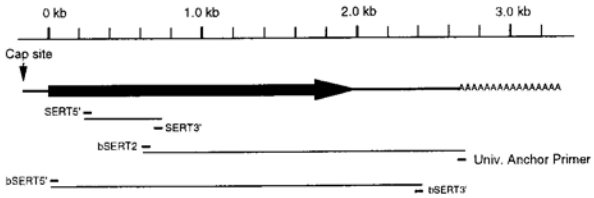

Fig. 1. Strategy for cloning a bSERT cDNA with RT-PCR. Initially, a 550-bp fragment from the coding region was cloned with primers SERT5′ and SERT3′, next the 3′ end of the cDNA was cloned with primers bSERT2 and a universal anchor primer, finally a cDNA containing the entire coding sequence, indicated by a bold arrow, and part of the 3′ untranslated region was cloned with primers bSERT5′ and bSERT3′.

2.4. 5-HT uptake

The pcDNA3 clones were used for transient expression of the bovine, human and rat serotonin transporters in HeLa cells. HeLa cells were cultured in DMEM media with 10% fetal calf serum and plated in 96 well plates to give 50% confluence. Each well was transfected with 70 ng DNA using the non-liposomal formulation Fugene-6 (Boehringer). Uptake assays were performed 40 h after transfection. The media was removed and the cells were washed twice with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3) containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBSCM). After washing, cells were incubated at 37°C for 2 min in PBSCM containing various concentrations of [^{3}H]-serotonin. Uptake was terminated by washing three times with ice-cold PBSCM, and the cells were lysed with 2M NaOH. The amount of accumulated [^{3}H] serotonin was determined by liquid scintillation counting in a Packard scintillation counter 1900CA. Specific uptake was determined as the difference between transfected and mock-transfected cells. Assuming Michaelis–Menten kinetics the data were plotted and analyzed by nonlinear least-squares curve fit (GraphPad Prism). Assays were carried out in triplicate and data on K_m and V_{max} are presented as mean values ± standard deviation from at least five independent transfections. In inhibition studies, 25 nM [^{3}H]-serotonin was incubated with increasing concentrations of inhibitor and incubated for 10 min at 20°C. Assays were carried out in triplicate and IC_{50} values are given as mean values ± standard deviation from at least three independent transfections.

In experiments to determine the sodium dependence, NaCl was replaced with either LiCl or choline chloride. To

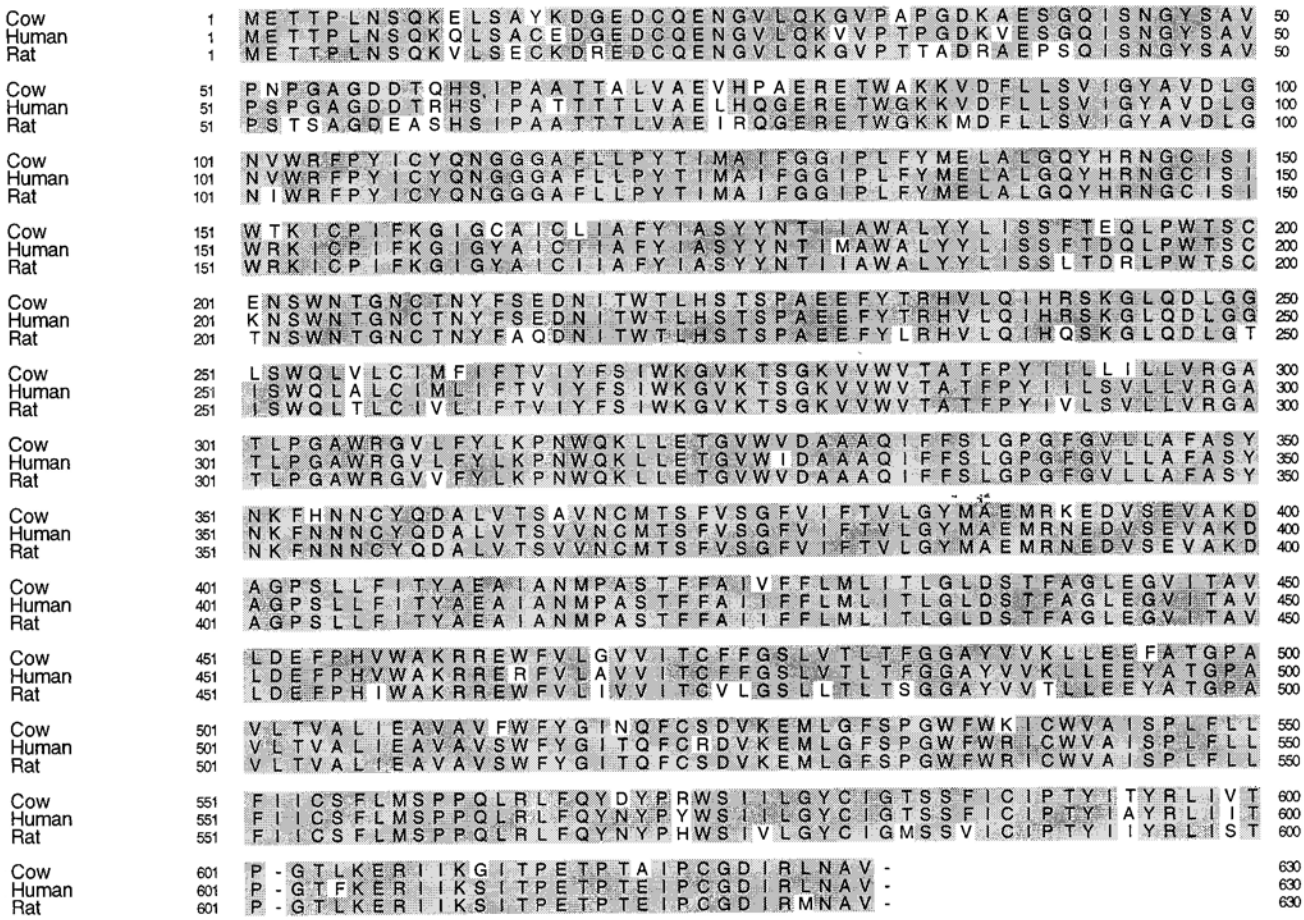

Fig. 2. Alignment of the translated sequences from bovine, human and rat SERT cDNA clones. Identical amino acids are shown in a grey background.

examine substrate specificity, cells were incubated with either 1 μM [^{3}H]-serotonin, 1 μM [^{3}H]-dopamine or 1 μM [^{3}H]-norepinephrine, respectively. All experiments were carried out in triplicate.

3. Determination of B_{max} on whole cells

The cocaine analogue RTI-55 (β-carbomethoxy-3β-(4-iodophenyl)tropane or β-CIT) was used for determination of B_{max} in transfected whole living cells. Cells were rinsed twice, prior to binding, in ice cold KRH binding buffer (5 mM Tris, 7.5 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM MgSO4, 1 mM ascorbic acid, 5 mM glucose, pH 7.1) and four times after binding in the same buffer. Binding was performed at 0°C for 1 h at 8 different concentrations of [^{125}I]-RTI-55. Ligand concentrations varied between 0.1–4 nM. Non-specific binding was determined by measuring binding to non-transfected cells. Assuming a single population of noninteracting binding sites, data were plotted and analyzed as described above. Assays were carried out in triplicate and data on B_{max} are presented as mean values ± standard deviation from at least four independent transfections.

4. Results and discussion

To obtain a SERT sequence from a new species we decided to clone a bovine SERT cDNA and to carry out the functional characterization of bSERT. Initially, a partial internal 550 bp fragment of bSERT was obtained by reverse transcription of mRNA from bovine placenta followed by PCR amplification and cloning. Primers were designed to match conserved regions in the coding sequence. The clone obtained was sequenced and the sequence was used to design a sense primer for amplification of the 3′ region using 3′ rapid amplification of cDNA ends (3′RACE). From the sequence of this clone, an antisense primer in the 3′ UTR was designed and together with a degenerate 5′ sense primer positioned right at the ATG start codon, a full length cDNA clone was obtained. The strategy is shown in Fig. 1. All the PCR amplifications were done using a proofreading polymerase to lower PCR artifacts and the full length cDNA was cloned independently six times. By alignment of the six sequences, a consensus sequence was obtained. The length of the 3′UTR was determined to be 670 bp and a polyA signal was located 550 bps downstream of the stop codon.

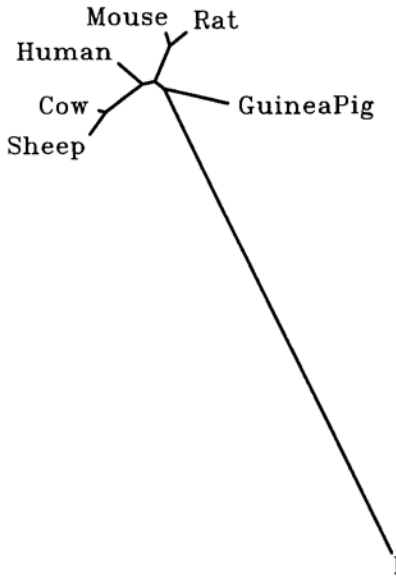

Fig. 3. Unrooted phylogenetic tree (PHYLIP version 3.57). Length of lines indicates relative evolutionary distances between species.

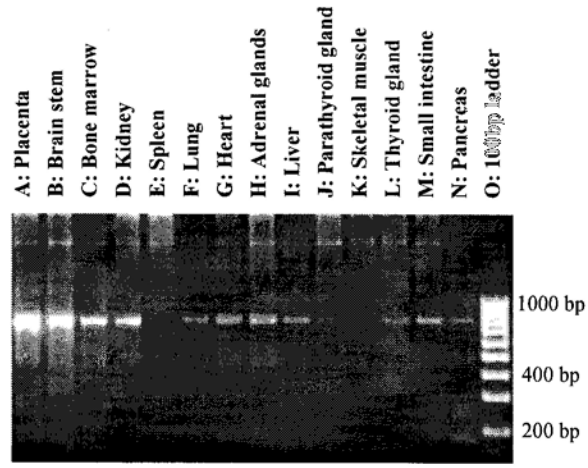

Fig. 4. RT-PCR amplification of RNA prepared from the different tissues indicated.

Sequencing of the cDNA revealed an open reading frame of 1890 base pairs resulting in a protein consisting of 630 amino acids. The translated sequence is aligned with rSERT and hSERT in Fig. 2. The sequence revealed the typical structure of sodium/chloride dependent transporters, and hydropathy plots showed the protein most likely contains 12 transmembrane domains like other transporters of this family and with N- and C-termini located intracellularly. bSERT has high homology to previously cloned SERTs with the highest homology to the ovine transporter. Fig. 3 shows an unrooted phylogenetic tree of presently known SERTs constructed by the software PHYLIP (Version 3.57). Drosophila is the most distant relative and the sheep and the cow the most closely related ones with only 11 of 630 amino acids differing (98% identity). Forty-four amino acids differ between bovine and human SERT (93% identity).

bSERT contains two potential *N*-glycosylation sites in the extracellular loop between TMD3 and TMD4. These sites are also present in SERT from other species, and in a

value for bSERT might be due to higher RNA stability or a higher efficiency during translation, folding, trafficking or it is caused by a greater protein stability.

In further characterization, uptake by bSERT was found to be sodium dependent as substitution of sodium with lithium or choline in the uptake buffer abolished uptake (data not shown). The substrate specificity was also investigated and uptake was found to be below the detection level for the most closely related substrates dopamine and norepinephrine (data not shown).

To establish a pharmacological profile of bSERT, a number of inhibition studies was carried out with serotonin selective reuptake inhibitors (SSRIs), tricyclic antidepressants and an amphetamine derivative (MDMA or ecstacy). The potency of the SSRIs citalopram, fluoxetine and paroxetine on bSERT was decreased when compared with rSERT and hSERT. Most notably, the IC_{50} value for citalopram was increased more than 10-fold compared to hSERT (Fig. 6, Table 2). The potency of MDMA on bSERT and hSERT is comparable and both were found to be slightly less sensitive than rSERT. The tricyclic antidepressants desipramine and imipramine were both less potent with bSERT compared to hSERT. Like bSERT, rSERT also is several fold less sensitive to imipramine compared to hSERT. In a recent study, this was found to be due to a divergence at amino acid position 586, which is a valine in rSERT and phenylalanine in hSERT [2]. In bSERT, however, this residue is also a phenylalanine, thus the increased resistance towards imipramine must be due to some of the other 44 divergent residues between bSERT and hSERT.

Table 2
IC_{50} values for uptake inhibition

	Bovine SERT (nM)	Human SERT (nM)	Rat SERT (nM)
Citalopram	255 ± 39	20.3 ± 7.6	12.3 ± 4.6
Fluoxetine	160 ± 2.5	41.0 ± 7.0	59 ± 6.1
Paroxetine	43 ± 3.0	18.1 ± 3.8	7.1 ± 2.1
Imipramine	146 ± 6.8	30.0 ± 8.9	202 ± 13
Desipramine	1435 ± 95	632 ± 46	1143 ± 220
MDMA	903 ± 178	819 ± 90	196 ± 52

Inhibition studies were carried out at a serotonin concentration of 25 nM and with increasing inhibitor concentrations. IC_{50} values are given as mean ± S.D. determined from three independent transfections. At each inhibitor concentration uptake assays were carried out in triplicate.

To summarise, bSERT, which is expressed in most bovine tissues, possesses high affinity uptake with increased K_m compared to rSERT and hSERT. Expression was higher for bSERT as both V_{max} and B_{max} increased, but the turnover number, V_{max}/B_{max} was conserved. Also, bSERT was found to be less sensitive than hSERT to several drugs. Therefore, among the 44 divergent residues, one or more might constitute other loci, in addition to phenylalanine 586, for binding of pharmacophoric elements of imipramine and SSRIs, and for decreasing substrate affinity.

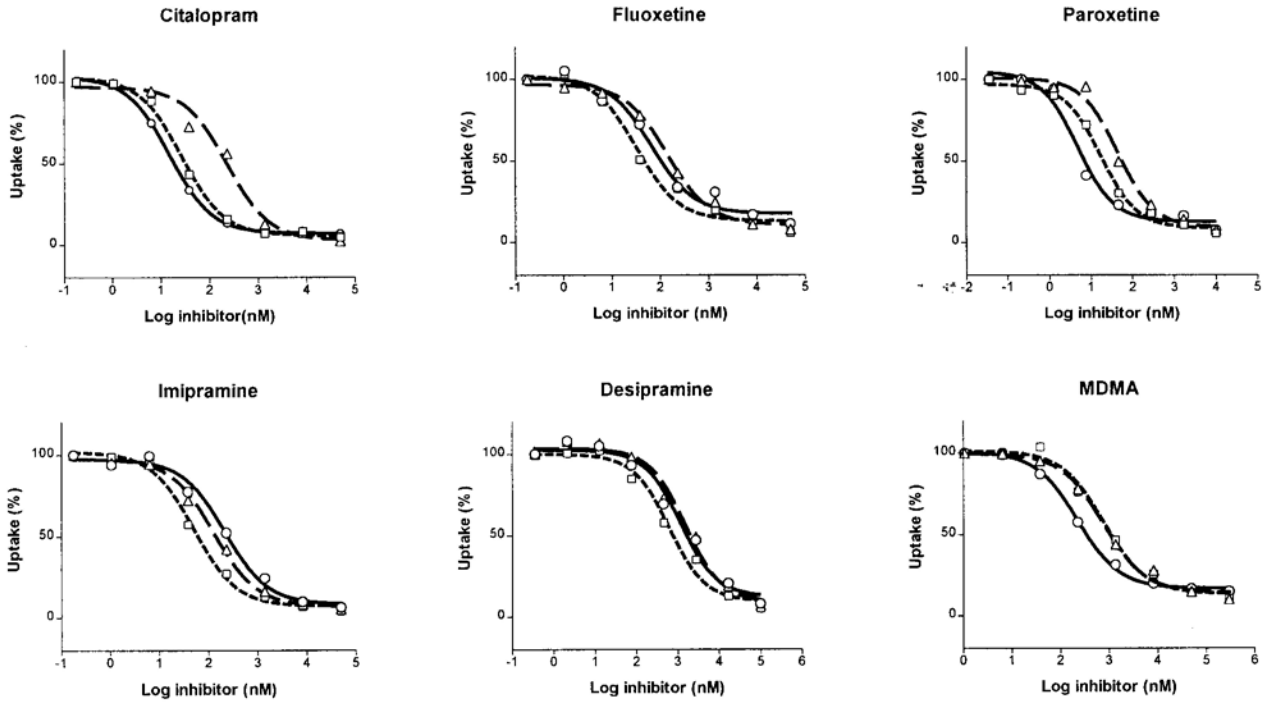

Fig. 6. Inhibition curves. Uptake is given as percentage of uptake level in the absence of inhibitors. △ bSERT, □ hSERT, ○ rSERT.

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