British Journal of Pharmacology (1999) 128, 51–60
http://www.stockton-press.co.uk/bjp

Recovery of dopamine neuronal transporter but lack of change of its mRNA in substantia nigra after inactivation by a new irreversible inhibitor characterized *in vitro* and *ex vivo* in the rat

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1 *In vitro*, the ability of DEEP-NCS {1-[2-(diphenylmethoxy)ethyl]-4-[2-(4-isothiocyanatophenyl)ethyl]-piperazine} to inhibit [^{3}H]-dopamine uptake by rat striatal synaptosomes was concentration-dependent and inversely related to the protein concentration. This inhibition was irreversible and resulted from changes in V_{max} and K_M. DEEP-NCS was less potent on noradrenaline, serotonin and choline transport.

2 One day after intrastriatal injections of DEEP-NCS (100 and 1000 pmol) in 20% dimethylsulphoxide, moderate decreases in the *ex vivo* dopamine uptake were observed in synaptosomes obtained from striatum injected with DEEP-NCS or solvent, and the contralateral uninjected striatum.

3 In similar conditions, 300 pmol DEEP-NCS in 45% 2 hydroxypropyl-γ-cyclodextrin – 0.5% dimethylsulphoxide solution sub-totally reduced *ex vivo* dopamine uptake and mazindol binding, and moderately decreased choline and serotonin transport. These reductions were specific to DEEP-NCS-injected striata. A clomipramine pretreatment (16 mg kg^{-1} i.p. 1 h before) was performed in following experiments, since it reduced the DEEP-NCS-elicited decrease in serotonin uptake without affecting other indices.

4 One day after intrastriatal injection, DEEP-NCS elicited similar dose-dependent decreases in *ex vivo* dopamine uptake and mazindol binding (ID_{50} = 6.9-8 ng striatum^{-1}). Changes in K_M and V_{max} for *ex vivo* dopamine transport produced by DEEP-NCS disappeared according to similar time-courses.

5 The $t_{½}$ for transporter recovery was 6.1 days. This value should correspond to its actual turnover rate *in vivo*, since no change in transporter mRNA level was observed in substantia nigra ipsilateral to 300 pmol DEEP-NCS-injected striatum.

6 The results indicate that DEEP-NCS behaves as a potent, quite selective, irreversible inhibitor of the DAT, *in vitro* and *in vivo*. Its use *in vivo* suggests that the physiological half-life of the rat striatal DAT is close to 6 days.

Keywords: Irreversible inhibitor; renewal; neuronal dopamine transporter; rat striatum; biogenic amine transporters; choline transporter; substantia nigra; mRNA levels; *in vitro*; *ex vivo*

Abbreviations: DA, dopamine; DAT, neuronal transporter of dopamine; DEEP, 1-[2-(diphenylmethoxy)ethyl]-4-[2-(azido-3-iodophenyl)ethyl] piperazine; DEEP-NCS, 1-[2-(diphenylmethoxy)ethyl]-4-[2-(4-isothiocyanatophenyl)ethyl]-piperazine, dihydrochloride monohydrate; DMSO, dimethylsulphoxide; EEDQ, N-ethoxy carbonyl-2-ethoxy-1,2-dihydroquinoline; GBR 12783, 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenyl-2-propenyl)-piperazine, dihydrochloride; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; 2HγCD, 2 hydroxypropyl-γ-cyclodextrin; 5-HT, serotonin; IC_{50}, inhibiting concentration 50%; ID_{50}, inhibiting dose 50%; *k*, rate constant for DAT degradation; K_M, concentration of DA which half-maximally stimulates DA transport; MR 14001, 1,4-bis{2-bis[(4-chlorophenyl) methoxy ethyl]} piperazine, dioxalate; MR 14503, 1-(2-diphenyl methoxy hexyl)-4-(3-phenyl-2-propenyl)-piperazine, dihydrochloride; NA, noradrenaline; PCR, polymerase chain reaction; *r*, rate constant for DAT production; RTI-76, 3β-(3*p*-chlorophenyl)tropan-2β-carboxylic acid, *p*-isothiocyanatophenylethyl ester HCl}; RTI-82, 3β-(*p*-chlorophenyl)tropan-2β-carboxylic acid, 4′ azido-3′ iodophenylethyl ester; SN, substantia nigra; $t_{½}$, half-life; V_{max}, maximal rate of transport; VTA, ventral tegmental area

Introduction

A number of irreversible inhibitors have been proposed for studying the dopamine neuronal transporter (DAT) or as antagonists of the stimulant properties of some abused agents. Phencyclidine derivatives such as metaphit and fourphit have been reported to produce, *in vitro*, an irreversible inhibition of the neuronal uptake of dopamine (DA) (Snell *et al*., 1987; Schweri *et al*., 1989) and of the specific binding of [^{3}H]-cocaine (Berger *et al*., 1986), [^{3}H]-methylphenidate (Schweri *et al*., 1987; 1989; 1992) and [^{3}H]-mazindol (Zimanyi *et al*., 1989) to the stimulant recognition site on the DAT. These derivatives, and especially metaphit, also recognize various receptors and ion channels (Zimanyi *et al*., 1989; Reith *et al*., 1991). Consequently, more selective irreversible antagonists for abused agents have been searched in other chemical series. A derivative of rimcazole, the 9-[3-(cis-3,5-dimethyl-4-(6-isothiocyanatohexyl)-1-piperazinyl) propyl]-carbazole, has been proposed as a specific blocker of the low affinity component of the

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cocaine binding site which could be related to σ sites (Husbands *et al*., 1997). Isothiocyanate derivatives of aryl 1-4 dialkylpiperazines, which irreversibly inhibit the [^{3}H]-DA uptake and the binding of [^{3}H]-methylphenidate or [^{3}H]-WIN 35,428 to the DAT *in vitro*, could constitute powerful cocaine antagonists (Deutsch *et al*., 1992; Deutsch & Schweri, 1994).

Photoaffinity probes of various chemical structures have been prepared as tools for studying the DAT (Grigoriadis *et al*., 1989; Thurkauf *et al*., 1991). Thus, the aryl 1-4 dialkylpiperazine compound 1-[2-(diphenylmethoxy)ethyl]-4-[2-(azido-3-iodophenyl)ethyl]piperazine (DEEP), its bis-(4-fluorophenyl) derivative (FAPP), and the 3β-phenyltropane compound 3β-(*p*-chlorophenyl)tropan-2β-carboxylic acid, 4′-azido-3′ iodophenylethyl ester (RTI-82) have been shown to recognize different but mutually exclusive binding sites on the DAT (Sallee *et al*., 1989; Patel *et al*., 1992; Vaughan & Kuhar, 1996; Vaughan *et al*., 1999). These data strengthened the hypothesis that inhibitors from the aryl 1–4 dialkylpiperazine series, the so-called GBR compounds, and cocaine derivatives occupy dissimilar but possibly overlapping binding sites on the DAT. Several other potentially irreversible inhibitors derived from cocaine and 3β-phenyltropane produce an *in vitro* (Lewin *et al*., 1992), wash-resistant blockade of the [^{3}H]-WIN 35,428 binding to the DAT (Boja *et al*., 1990; 1991; Carroll *et al*., 1992). The inactivation of the DAT resulting from a stereotaxic injection of the most potent of these irreversible inhibitors, the 3β-(3*p*-chlorophenyl)tropan-2β-carboxylic acid, *p*-isothiocyanatophenylethyl ester HCl (RTI-76), has allowed an estimate of the $t_{½}$ value for DAT recovery in rat striatum to be 6 days (Fleckenstein *et al*., 1996).

Recent work concerning inactivation of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors in rat brain gave evidence that an irreversible blockade of a receptor can produce an increase in its mRNA level (Raghupathi *et al*., 1996a,b). The time-course and the intensity of this increase varied as a function of the cerebral area which was considered and as a function of the magnitude of the initial inactivation (Raghupathi *et al*., 1996a,b). Thus, an irreversible blockade of a low to moderate proportion of the 5-HT$_{1A}$ population produced no change in mRNA levels, whereas inactivation of more than 90% of the receptor population resulted in a strong increase in 5-HT$_{1A}$ mRNA levels (Raghupathi *et al*., 1996b). By analogy, inactivation of the DAT could increase its mRNA level and, consequently, modify its rate of recovery compared to that observed under physiological conditions. On the other hand, it has previously been suggested that the specificity of the agent used for receptor inactivation can influence the rate of receptor recovery. The half-life of 5-HT$_{1A}$ sites in rat hippocampus estimated after an irreversible blockade by N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), which also blocks several other types of receptor, was twice as long as that observed after inactivation by a more selective 5-HT$_{1A}$ alkylating agent, i.e. 2.3 days (Hamon *et al*., 1988; Gozlan *et al*., 1994). In the same way, the half-life for D$_2$ receptor recovery was lower after inactivation by a selective irreversible agent (170 h) than after EEDQ (77 h) (Xu *et al*., 1991).

Thus, the present work was carried out to estimate the rate of DAT recovery after inactivation by intrastriatal injection of a GBR derivative, the 1-[2-(diphenylmethoxy)ethyl]-4-[2-(4-isothiocyanatophenyl) ethyl]-piperazine, dihydrochloride (DEEP-NCS) (Figure 1). This inhibitor belongs to a chemical class displaying a pharmacological profile different from that of RTI-76. Furthermore, these agents may recognize different binding sites on the DAT (Sallee *et al*., 1989; Patel *et al*., 1992; Vaughan & Kuhar, 1996; Vaughan *et al*., 1999). A quantification of DAT mRNA in substantia nigra (SN) was conducted to ascertain that inactivation of DAT present in nerve terminals membranes did not modify the mRNA level in the corresponding cell bodies. The ability of DEEP-NCS to affect DAT function and availability was quantified by studying [^{3}H]-DA uptake and [^{3}H]-mazindol binding. According to previous works, [^{3}H]-mazindol recognizes a single binding site on the DAT (Javitch *et al*., 1984; Dersch *et al*., 1994; Sonders *et al*., 1997). *Ex vivo* experiments were performed after a preliminary demonstration of the *in vitro* irreversible character of the DA uptake inhibition by DEEP-NCS.

Methods

Synthesis of DEEP-NCS

1-chloro-2-(diphenylmethoxy)ethane **1**, 1-[2-(diphenylmethoxy)ethyl]piperazine **2**, 1-[2-(diphenylmethoxy)ethyl]-4-[2-(4-nitrophenyl) ethyl] - piperazine **3** and 1 - [2 - (diphenylmethoxy) ethyl]-4-[2-(4-aminophenyl)ethyl]-piperazine **4** were prepared

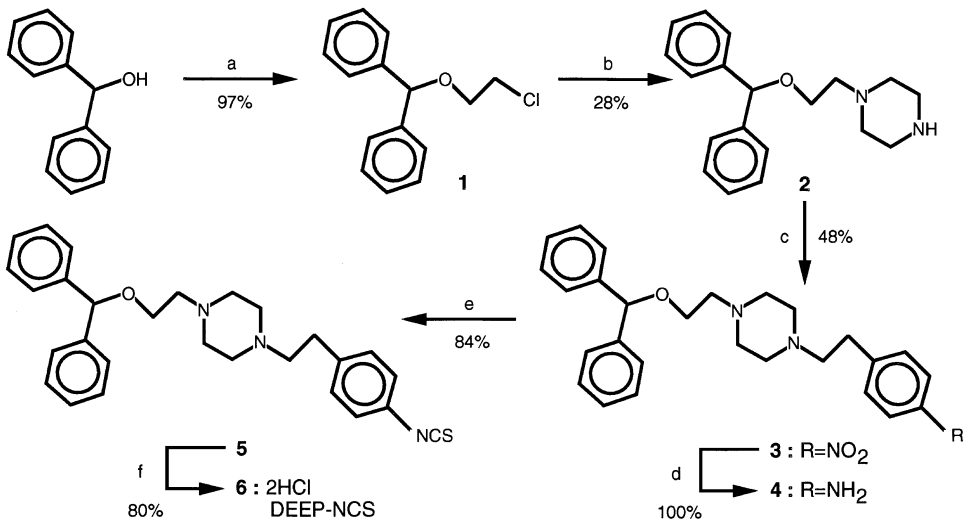

Figure 1 Structure and general synthetic route of DEEP-NCS. (a) $HOCH_2CH_2C1$, H_2SO_4, CH_2C1_2, RT, 1 h 45; (b) Piperazine, K_2CO_3, toluene, reflux, 5 h; (c) $4\text{-}NO_2PhCH_2CH_2Br$, K_2CO_3, EtOH, reflux, 3 h; (d) H_2, Pd/C 10%, EtOH, RT, 1 atm, 16 h; (e) $CSC1_2$, $CHC1_3$, H_2O, RT, 25 min; (f) HC1 gas, EtOH, RT.

according to procedures described by Van der Zee *et al.* (1980) (Figure 1). 1-[2-(diphenylmethoxy)ethyl]-4-[2-(4-isothiocyanatophenyl)ethyl]-piperazine **5** was prepared as follows: a suspension of compound **4** (0.70 g, 1.68 mmol) and $NaHCO_3$ (0.99 g, 11.79 mmol) in 10 ml of water and 30 ml of chloroform was stirred at room temperature. Thiophosgene (0.171 ml, 2.24 mmol) was added *via* syringe and the mixture stirred 25 min. The chloroform layer was separated, combined with a single extraction with 15 ml of chloroform and the aqueous phase, dried over $MgSO_4$ and evaporated. The residue was dissolved in a solution of diethyl ether: pentane (1 : 1) and evaporated, giving compound **5** (0.65 g, 84% yield) as a crude pale yellow solid m.p.: 67–68°C; ^{1}H n.m.r. (200 MHz, $CDCl_3$): 2.35–2.95 (m, 14H, CH_2), 3.59 (t, 2H, OCH_2, J = 6.0 Hz), 5.35 (s, 1H, BnzH), 7.05–7.40 (m, 14H, ArH); ^{13}C n.m.r. (50 MHz, $CDCl_3$): 142.1, 139.8, 134.9, 129.7, 128.9, 128.3, 127.4, 126.9, 125.8, 83.9, 66.8, 59.6, 57.8, 53.4, 52.9, 33.0.

Preparation of the hydrochloride monohydrate salt **6**: to a solution of compound **5** (0.22 g, 0.48 mmol) in 10 ml of ethanol was bubbled at room temperature dry gaseous HCl until a white precipitate appeared. Then the mixture was filtered, the precipitate was collected and dried at 60°C under vacuum. Compound **6** (0.205 g, 80% yield) was obtained as a white solid. m.p.: 194–196°C; ^{1}H n.m.r. (200 MHz, $CDCl_3$): 1.70 (s large, 2H, NH^+), 3.10–4.25 (m, 16H, CH_2), 5.45 (s, 1H, BnzH), 7.10–7.45 (m, 14H, ArH); FAB-MS (m/e, per cent intensity): 458 (M + 1-2HCl, 32), 167 (100), 155 (51) 137 (37), 136 (37), 93 (32); h.p.l.c.: column C_{18} Nova Pak HR (Waters) 0.8 × 10 cm, eluent CH_3CN:H_2O (+0.1% TFA) = 60 : 40, flow: 2 ml min^{-1}, UV detection at λ = 254 nm, RT = 2.57 min, purity 98.1%; elemental analysis: $C_{22}H_{31}N_3OS$. 2HCl. $1H_2O$; found: %C 59.57 %N 7.44 %H 6.25; theoretical: %C 60.09 %N 7.42 %H 5.97.

[^{3}H]-DA uptake

All procedures necessary to prepare synaptosomal suspensions were done at 0–2°C. Male Sprague Dawley rats (150–200 g), purchased from Charles River (Saint Aubin lès Elbeuf, France), were killed by decapitation and the striata were dissected out and homogenized with 12 up-and-down strokes of a Teflon-glass homogenizer (800 r.p.m.) in 10 volumes (w/v) of ice-cold 0.32 M sucrose. The nuclear material was removed by centrifugation (1000 × *g*, 10 min) and the supernatant was stored. The pellet was resuspended in an equal volume of sucrose and recentrifuged. The two supernatants were combined and constituted a crude synaptosomal suspension S_1 which was used for uptake and binding experiments.

Aliquots (50 μl) of S_1 were preincubated for 5 min at 37°C in a Krebs Ringer medium containing (mM): NaCl 109, KH_2PO_4 1, $CaCl_2$ 1, $NaHCO_3$ 27, glucose 5.4, pH 7.4. The incubation was continued for 5 min, in the same medium, containing 10 nM [^{3}H]-DA (1 ml final volume). The reaction was stopped by adding 3 ml of ice-cold incubation medium containing 100 μM cocaine and immediate centrifugation (7000 × *g*, 10 min, 4°C). The pellet was washed with 1 ml of the latter medium and centrifuged (7000 × *g*, 15 min). The final pellet was sonicated in 250 μl distilled water and aliquots of the homogenate were used for the determination of radioactivity and protein concentrations. The radioactivity was determined by liquid scintillation spectrometry in 5 ml Optiphase Highsafe II® with 33–36% counting efficiency. Protein concentrations were determined according to the method of Lowry *et al.* (1951), using bovine serum albumin as standard. The specific uptake of DA was defined as the difference between the total uptake at 37°C and non-specific accumulation observed at 0°C in the presence of 100 μM cocaine.

Saturation experiments were performed in the presence of 30–500 nM [^{3}H]-DA concentrations, and for a 1 min incubation period which was within the initial linear phase of DA accumulation.

Washing procedures

Aliquots of S_1 corresponding approximately to 3 mg protein were incubated at 37°C for 5 min in 10 ml of Krebs Ringer medium, containing a 1–3 μM concentration of inhibitor when necessary. Then, a part of the preparation was used for quantification of the [^{3}H]-DA uptake according to aforementioned procedures. The remaining part was diluted by 40 ml of ice-cold Krebs Ringer medium and centrifuged (45,000 × *g*, 10 min, 4°C). The pellet was resuspended in 1.8–2 ml Krebs Ringer medium and an aliquot allowing to obtain a protein concentration of 50–100 μg per assay was spared for uptake quantification. This washing procedure was repeated four times.

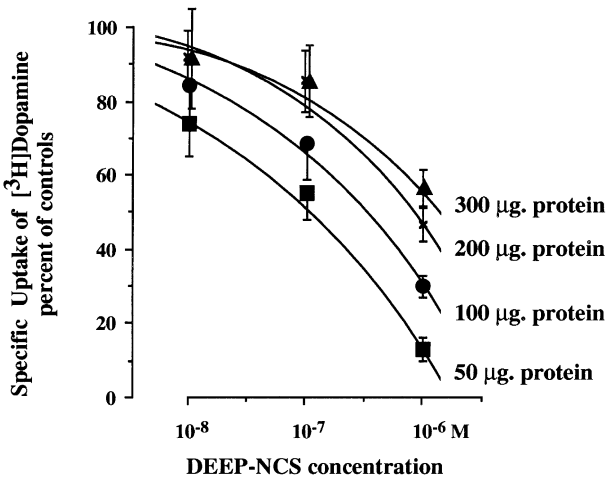

Figure 2 Effect of DEEP-NCS on the *in vitro* uptake of [^{3}H]-DA. Aliquots of synaptosomal suspensions obtained from rat striatum (50–300 μg protein) were incubated in the presence of DEEP-NCS as described in Methods. IC_{50} values were 0.13 ± 0.015 μM and 0.34 ± 0.024 μM for 50 and 100 μg protein respectively. Data are means ± s.e.mean of three experiments performed in duplicate.

Table 1 *In vitro* inhibition of the neuronal uptake of amines by DEEP-NCS

	Specific uptake (pmol mg protein^{-1} 5 min^{-1})			
	[^{3}H]-NA	[^{3}H]-DA	[^{3}H]-5-HT	[^{3}H]-choline
Controls	6.42 ± 0.32	13.55 ± 0.45	15.8 ± 0.57	3.12 ± 0.06
DEEP-NCS				
1 μM	3.27 ± 0.33	2.80 ± 0.25	7.64 ± 0.42	3.00 ± 0.06
3 μM	2.32 ± 0.11			
10 μM	1.74 ± 0.12			
30 μM	1.24 ± 0.15			

Aliquots of crude synaptosomal suspensions obtained from rat hypothalamus ([^{3}H]-NA) or striatum (other amines) were incubated as described in Methods. The specific uptake was defined as the difference between the total uptake at 37°C and non-specific accumulation at 0°C in the presence of 0.3 μM desipramine ([^{3}H]-NA), 0.1 mM cocaine ([^{3}H]-DA), 1 μM fluoxetine ([^{3}H]-5-HT) or 0.1 mM hemicholinium ([^{3}H]-choline). Data are means ± s.e.mean of four experiments performed in duplicate. [^{3}H]-NA transport experiments were performed in the presence of 30 nM GBR 12783 in order to block NA transport by DAT. This resulted in a 23% decrease in specific uptake.

Uptake of [^{3}H]-choline, [^{3}H]-serotonin (5-HT) and [^{3}H]-noradrenaline (NA)

Uptake experiments were performed on aliquots of crude synaptosomal suspensions obtained from rat hypothalamus ([^{3}H]-NA) or striatum (other amines) according to general experimental procedures described for [^{3}H]-DA uptake studies. Non-specific accumulation at 0°C was quantified in the presence of 0.3 μM desipramine for [^{3}H]-NA, 1 μM fluoxetine for [^{3}H]-5-HT or 0.1 mM hemicholinium for [^{3}H]-choline. [^{3}H]-NA uptake assays were performed in the presence of 30 nM 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenyl-2-propenyl)-piperazine, dihydrochloride (GBR 12783) in order to block NA transport by DA nerve terminals present in hypothalamic preparations.

[^{3}H]-mazindol binding

The crude synaptosomal suspension S_1 was sonicated for 5 s (microprobe diameter 3 mm; Sonics & Materials, Inc., Danbury, CT, U.S.A.) in 15 volumes of a 10 mM Na^+ medium containing (mM concentrations): 0.2 NaH_2PO_4, 9.8 $NaHCO_3$, pH 7.4. Aliquots of membrane suspensions (50–100 μg protein; Lowry *et al*., 1951) were incubated at 0°C for 2 h in a 10 mM Na^+ medium (final volume, 0.5 ml) containing [^{3}H]-mazindol (2.5 nM final concentration). Incubations were stopped by filtration through GF/B filters previously soaked for at least 1 h in 0.5% polyethyleneimine. Each tube was rinsed once and filters were rinsed once again with 3 ml of ice-cold incubation medium. Filters were counted for radioactivity in 5 ml Optiphase Hisafe II®. Non specific binding was determined in the presence of 100 μM cocaine.

Striatal injections of DEEP-NCS

Rats were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.) and placed in a stereotaxic apparatus (David Kopf) to permit injection of 10 μl of a DEEP-NCS solution into one striatum (A: +8 mm, L: 3.5 mm, D: 3.5 mm according to Albe-Fessard *et al*., 1971) over 10 min.

Quantification of DAT mRNA by polymerase chain reaction (PCR) assays

Rats were killed by decapitation and SN ipsilateral to injected striata were dissected out. mRNA for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as standard. For each assay, total RNA was extracted from two pooled SN, using the RNA InstaPure System (Eurogentec Belgium) according to the manufacturer's procedure, then briefly dried under vacuum, resuspended in ribonuclease-free water and quantified by measuring absorbance at 260 nm. The first strand of cDNA was synthesized by reverse transcription. A 30 μl reverse transcription reaction mixture containing 2 μg total RNA (heated at 65°C for 10 min and then quenched on ice for 5 min), reverse transcription buffer (50 mM Tris/HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 5 mM dithiothreitol, 1 mM deoxynucleosides triphosphate, 33 U RNasin inhibitor, 250 pmol $pd(N)_6$ and 400 U Moleney-murine leukemia virus reverse transcriptase was incubated at 37°C for 1 h, heated to 95°C for 5 min and then quick-chilled on ice.

Primers for PCR of DAT were obtained from Genosys, and were selected from the cDNA encoding for rDAT: 5′-TCCCTGACAAGCTTCTCC-3′ (nucleotides 1057–1074) and reverse 5′-GCCAGGACAATGCCAAGA-3′ (nucleotides 1344–1361). Primers for G3PDH were obtained from Clontech. PCR was performed in a Perkins-Elmer/Cetus thermal cycler on 2 μl of the cDNA reaction mixture at final concentrations of PCR buffer, 2.5 mM $MgCl_2$, 200 mM deoxynucleosides triphosphate, 40 pmol of each of the downstream (5′) and upstream (3′) primers and 2.5 U Taq DNA polymerase in a total volume of 50 μl. The amplification profile involved four linked files as follows; 5 min at 94°C for 1 cycle; 1 min at 94°C, 1 min at 60°C and 1 min at 72°C for 28 cycles for DAT or 26 cycles for standard; and finally 8 min at 72°C for 1 cycle. The PCR was carried out at different cycle numbers to obtain results higher than the limit of detection, but below the PCR plateau. A 10 μl aliquot of each amplified PCR sample (DAT and standard) was electrophoresed on a 1.5% agarose gel. The gel was stained with ethidium bromide and photographed for densitometry analysis. The expected size of the amplified fragments was evaluated using the 1 kb marker (300 base pairs for DAT and 1000 base pairs for standard).

Densitometry analysis was performed as follows: a black and white CCD camera (IVC 500 BC) allowed image acquisition using a Matrox (IDS 542) placed in an IBM AT computer. Grey levels were scored using PCSCOPE 2:0 image analysis software (I2S, Bordeaux, France).

Drugs

GBR 12783 and two other uptake inhibitors, 1,4-bis{2-bis [(4-chlorophenyl)methoxyethyl]} piperazine, dioxalate (MR 14001) and 1-(2-diphenyl-methoxyhexyl)-4-(3-phenyl-2-propenyl)-piperazine, diHCl (MR 14503) were synthesized by Professor Robba (Caen, France) (Lancelot *et al*., 1990; 1993).

[^{3}H]-dopamine (7-25 Ci $mmol^{-1}$), [^{3}H]-noradrenaline (14.7 Ci $mmol^{-1}$), [^{3}H]-choline (83 Ci $mmol^{-1}$), [^{3}H]-serotonin (15.2 Ci $mmol^{-1}$) were purchased from Amersham (Les Ulis, France). [^{3}H]-mazindol (24.8 Ci $mmol^{-1}$) was purchased from NEN (Les Ulis, France). The following drugs were

Table 2 Effect of washes on inhibition of [^{3}H]-DA uptake produced by DEEP-NCS, nomifensine, MR 14001 and MR 14503

Number of washes	*Specific uptake of [^{3}H]-DA* (pmol mg protein^{-1} 5 min^{-1}) *in*		
	Controls	1 μM *DEEP-NCS*	1 μM *Nomifensine*
0	16.01±1.85	2.52±0.10 (16%)	2.34±0.29 (14.5%)
1	14.03±0.14	2.09±0.07 (15%)	13.02±0.96 (93%)
2	11.72±1.39	2.06±0.64 (19%)	11.70±1.20 (100%)
3	10.71±1.22	2.46±0.54 (23%)	11.11±0.66 (106%)
4	8.44±0.96	2.10±0.61 (24%)	9.34±0.68 (112%)
	Controls	3 μM *MR14001*	3 μM *MR14503*
0	17.66±0.86	3.45±0.08 (20%)	2.40±0.09 (14%)
1	16.40±1.15	10.06±1.00 (61%)	8.77±0.50 (54%)
2	15.36±1.10	13.25±1.00 (86%)	12.70±0.60 (83%)
3	13.99±1.10	12.70±1.16 (90%)	13.27±1.10 (95%)
4	12.85±1.38	12.11±1.53 (94%)	12.44±1.44 (97%)

Aliquots of nerve endings suspensions obtained from rat striatum were incubated at 37°C for 5 min, in the presence of 1 μM DEEP-NCS, 1 μM nomifensine, 3 μM MR14001 or 3 μM MR14503. A part of this preparation was removed for a [^{3}H]-DA uptake quantification and the remainder was centrifuged and washed one to four times with 40 ml of ice-cold Krebs Ringer medium before testing for uptake. Percentages of remainding uptake are indicated between brackets. Data are means±s.e.mean of 3–4 experiments performed in duplicate.

donated by manufacturers: nomifensine maleate (Hoechst, L'Aigle, France), desipramine hydrochloride and clomipramine hydrochloride (Ciba Geigy, Huningue, France). Other drugs were from commercial sources.

For *in vitro* experiments, a 10 mM solution of DEEP-NCS was prepared in dimethylsulphoxide (DMSO) and then diluted in water (1 mM) and incubation medium (10 μM). Except when indicated in the text, 20 mM DEEP-NCS for intrastriatal injection was prepared in DMSO and then diluted in sterile water, 2 hydroxypropyl-γ-cyclodextrin (2HγCD) in water and DMSO in order to obtain final dilutions in 45% 2HγCD- 0.5% DMSO solutions. These solutions were prepared in glass tubes. 10 mM solutions of MR14001 and MR14503 were prepared in DMSO (50% in water), and then diluted in water (1 mM) and incubation medium (100 μM).

Statistics and calculations

Geometric means and 95% confidence limits were calculated for K_M and V_{max} values. ID_{50} and IC_{50} values (doses and concentrations of DEEP-NCS inhibiting 50% of the control uptake) were calculated by non-linear regression analysis of the specific uptake (Ligand, Biosoft, Cambridge, U.K.). The significance of changes was tested with a Dunnett's *t*-test since different treatments were compared with a common control group.

After irreversible blockade of the DAT, transporter repopulation kinetic was modelled to the equation given by Mauger *et al*. (1982):

$$[T_t] = r/k(1 - e^{-kt})$$

where $[T_t]$ is the transporter concentration at time t, r is the rate constant for DAT production and k is the rate constant of DAT degradation. The use of this equation is based on the assumptions that (1) DAT production takes place at a constant rate (r) and (2) the rate of DAT degradation (k) is proportional to DAT concentration. As time of repopulation approaches infinity, the term e^{-kt} approaches zero and $[T_t]$ approaches $[T_{ss}]$, i.e. the concentration of transporter at steady state. Hence, $[T_{ss}] = r/k$. By substitution into and after logarithmic transformation, the equation can be written

$$kt = \ln[T_{ss}]/([T_{ss}] - [T_t]).$$

The half-life of DAT recovery ($t_{½}$) was calculated according to the equation $t_{½} = 0.639/k$. The transporter production rate (r) was estimated from the time-course of V_{max} recovery, assuming a transporter density of 10 pmol mg protein^{-1} which corresponds to 250 pmol g striatum^{-1}.

Results

In vitro *experiments*

DEEP-NCS inhibited [^{3}H]-DA uptake by crude synaptosomal suspensions from rat striatum in a concentration-dependent manner (Figure 2). The intensity of this inhibition was inversely related to the protein concentration in assays: a 50% inhibition was provoked by 0.13 ± 0.015 μM DEEP-NCS for 50 μg protein in a 1 ml incubation volume, when it was $\geqslant$to 1 μM for 200–300 μg protein (Figure 2). The uptake inhibition resulted from mixed changes in V_{max} and K_M. So, V_{max} for the specific uptake in control suspensions (212 [177–251] pmol mg protein^{-1} min^{-1}) was significantly reduced to 176 [151–208] ($t = 3.4$; $P < 1\%$) and 158 [129–191] ($t = 5.1$; $P < 0.1\%$) pmol mg protein^{-1} min^{-1} by 10 nM and 100 nM

Table 3 Per cent inhibition of the uptake of [^{3}H]-DA, [^{3}H]-5-HT and [^{3}H]-choline and the binding of [^{3}H]-mazindol measured *ex vivo*, 24 h after a unilateral injection of DEEP-NCS in the rat striatum

	No pretreatment		*Clomipramine pretreatment*	
	Injected striatum	*Contralateral striatum*	*Injected striatum*	*Contralateral striatum*
Solvent: 20% DMSO solution				
[^{3}H]-Dopamine uptake				
Solvent alone	13±3	15±5	nt	nt
DEEP-NCS 100 pmol	30±4	36±2	nt	nt
DEEP-NCS 1000 pmol	45±7	48±6	nt	nt
Solvent: 45% 2HγCD solution containing 0.5% DMSO				
[^{3}H]-Dopamine uptake				
Solvent alone	1±5	0±3	−1±2	0±1
DEEP-NCS 300 pmol	96±7	−1±2	97±2	2±3
[^{3}H]-Serotonin uptake				
Solvent alone	2±6	0±5	8±2	10±1
DEEP-NCS 300 pmol	39±5	3±4	22±3	11±3
[^{3}H]-Choline uptake				
Solvent alone	1±3	0±6	3±1	−2±2
DEEP-NCS 300 pmol	12±4	1±6	11±3	−2±2
[^{3}H]-Mazindol binding				
Solvent alone	4±2	−2±1	4±4	0±2
DEEP-NCS 300 pmol	93±12	1±3	94±4	0±2

A stereotaxic injection (10 μl) was performed in one rat striatum. One day later, binding of [^{3}H]-mazindol and uptake of [^{3}H]-DA, [^{3}H]-5-HT and [^{3}H]-choline were studied on preparations obtained from either injected striata or contralateral striata. Data are expressed as per cent of inhibition of control values obtained in naive rats, and they correspond to means±s.e.mean of 3–4 experiments performed in duplicate. The different indices were quantified on suspensions obtained from striata injected with solvent (20% DMSO or 45% 2 HγCD solution containing 0.5% DMSO), or solution of DEEP-NCS in solvent. The intrastriatal injections were performed either in naive rats (no pretreatment) or in rats pretreated i.p., 1 h before, with clomipramine (16 mg kg^{-1}) or saline (controls). The [^{3}H]-DA uptake in controls for the first set of assays corresponds to 8.7±0.1 pmol mg protein^{-1} 5 min^{-1}. For other sets of assays, control values of [^{3}H]-DA, [^{3}H]-5-HT and [^{3}H]-choline and for binding of [^{3}H]-mazindol correspond to 1) 12.6±0.5 pmol mg protein^{-1} 5 min^{-1}, 8.0±0.5 pmol mg protein^{-1} 5 min^{-1}, 1.3±0.1 pmol mg protein^{-1} 5 min^{-1}, and 1.6±0.1 pmol mg protein^{-1}, respectively for not pretreated animals, and 2) 11.9±0.4 pmol mg protein^{-1} 5 min^{-1}, 5.3±0.2 pmol mg protein^{-1} 5 min^{-1}, 0.8±0.03 pmol mg-protein^{-1} 5 min^{-1} and 1.6±0.04 pmol mg protein^{-1}, respectively for clomipramine pretreated rats. nt, not tested.

DEEP-NCS respectively (geometric means and 95% confidence limits of four experiments performed in duplicate). In the same way, the K_M value in controls (210 [167–258] nM) was significantly enhanced to 284 [263–307] ($t=4.8$; $P<0.1\%$) and 370 [317–426] nM ($t=10.3$; $P<0.1\%$) by 10 nM and 100 nM DEEP-NCS respectively.

The inhibitor affected the neuronal uptake of other amines to a lesser extent. A 1 μM DEEP-NCS concentration which blocked 81% of the [^{3}H]-DA uptake reduced the specific transport of [^{3}H]-5-HT and [^{3}H]-choline in crude synaptosomal suspensions from rat striatum by 52 and 4% respectively (Table 1). DEEP-NCS also blocked [^{3}H]-NA uptake by hypothalamic synaptosomal suspensions in a concentration-dependent manner; a 50% blockade was observed for 1 μM DEEP-NCS (Table 1).

The irreversible character of the DA transport inhibition elicited by DEEP-NCS was demonstrated in washing experiments performed in conditions allowing the dissociation of reversible inhibitors of similar affinity for DAT, nomifensine, as a reference inhibitor, and two compounds structurally related to DEEP-NCS, MR 14001 and MR 14503 (Lancelot *et al*., 1990; 1993). As shown in Table 2, incubation of a crude synaptosomal suspension from rat striatum with a 1–3 μM concentration of inhibitor induced a 80–86% decrease in DA uptake. Repeated washes resulted in an easy and total restoration of the DA transport in suspensions incubated with nomifensine, MR 14001 and MR 14503. In contrast, no significant decrease in uptake inhibition was observed in suspensions treated with DEEP-NCS ($t\leqslant0.93$; NS). It is noteworthy that the washing procedure itself had some impact on the specific uptake of [^{3}H]-DA: a 27–47% reduction in transport was observed in controls between unwashed and four times washed synaptosomal suspensions (Table 2).

In vivo *inactivation*

Effects of stereotaxic injection of DEEP-NCS into the rat striatum on DAT availability were determined by an *ex vivo* quantification of DA transport and [^{3}H]-mazindol binding. In a first set of experiments, DEEP-NCS was injected as a solution in 0.9% NaCl containing 20% DMSO. A unilateral stereotaxic injection of 10 μl of solvent resulted, 1 day later, in a 13–15% decrease in the *ex vivo* [^{3}H]-DA uptake studied in synaptosomal suspensions prepared from either injected striata or control contralateral striata (Table 3). DEEP-NCS (100–1000 pmol) produced a moderate decrease (30–50%) in the *ex vivo* DA transport by crude synaptosomal suspensions obtained from either the injected striatum or the uninjected control (Table 3).

Striatal injections of a solution of DEEP-NCS in 45% 2HγCD containing 0.5% DMSO gave different results (Table 3). One day after administration, 300 pmol DEEP-NCS caused a major decrease in the *ex vivo* [^{3}H]-DA uptake and [^{3}H]-mazindol binding in preparations obtained from injected striata. In the same suspensions, the *ex vivo* uptake of [^{3}H]-5-HT and [^{3}H]-choline was reduced by 39 and 13% respectively (Table 3). In these experimental conditions, none of the studied parameters was affected in membrane and synaptosomes suspensions obtained from either the solvent-injected striata or from striata opposite to injected striata (Table 3).

Pretreatment of rats with clomipramine (16 mg kg^{-1}, i.p.), 1 h before the stereotaxic injection of DEEP-NCS, reduced from about 40% to 10–15% the intensity of the *ex vivo* inhibition of [^{3}H]-5-HT uptake measured 24 h after DEEP-NCS (Table 3). This pretreatment also decreased by 10% the *ex vivo* [^{3}H]-5-HT uptake in suspensions obtained from either solvent-injected striata or control contralateral striata (Table 3). On the contrary, the intensity of the decreases in the *ex vivo* [^{3}H]-mazindol binding and [^{3}H]-DA and [^{3}H]-choline uptakes was fully preserved and no change in these indices was observed in suspensions obtained from either solvent-injected striata or contralateral striata (Table 3). Consequently, the same pretreatment regimen was used in all subsequent experiments.

Table 4 Dose-response effects of DEEP-NCS on *ex vivo* [^{3}H]-DA uptake and [^{3}H]-mazindol binding

Dose of DEEP-NCS/ striatum		*Specific uptake of [^{3}H]-dopamine*	*Specific binding of [^{3}H]-mazindol*
pmol	*ng*	(pmol mg protein^{-1})	(fmol mg protein^{-1})
Solvent –	–	13.7±1.18	1363±22
10	5.3	7.50±0.28 (56%)	746±30 (55%)
30	15.9	4.26±0.34 (31%)	459±11 (34%)
100	53	2.03±0.13 (15%)	255±13 (19%)
300	159	0.89±0.09 (6.5%)	135±7 (10%)
1000	530	0.61±0.10 (4.5%)	55±5 (4%)

A unilateral injection of 10 μl of solvent (45% 2HγCD solution containing 0.5% DMSO) or 1–100 μM solutions of DEEP-NCS was performed in the striatum, 1 h after pretreatment of animals by 16 mg kg^{-1} clomipramine (i.p.). Rats were sacrificed 1 day later and [^{3}H]-DA uptake and [^{3}H]-mazindol binding quantifications were performed as described in Methods. Data are means±s.e.mean of four experiments performed in duplicate. Percentages of remaining process are indicated between parenthesis.

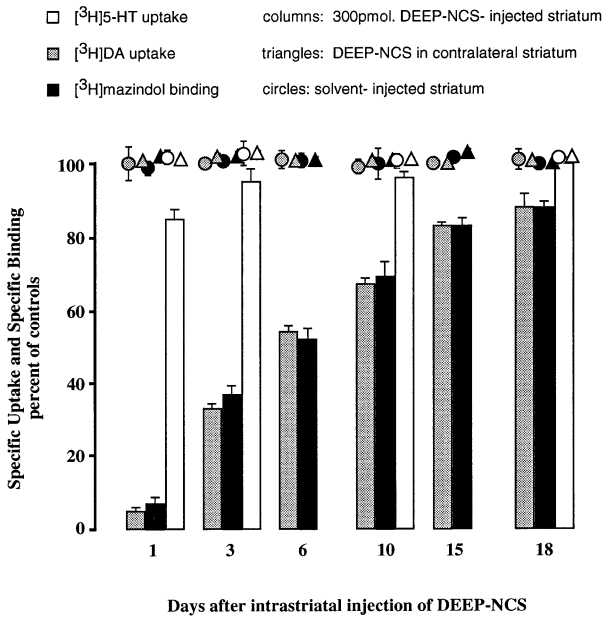

Figure 3 Time course (days) of the effect of an intrastriatal injection of 300 pmol DEEP-NCS on [^{3}H]-mazindol binding and neuronal uptake of [^{3}H]-DA and [^{3}H]-5-HT measured *ex vivo*. Unilateral striatal injections (10 μl) were performed 1 h after pretreatment of animals with 16 mg kg^{-1} clomipramine (i.p.). Rats were sacrificed and [^{3}H]-DA uptake (grey symbols), [^{3}H]-5-HT uptake (white symbols) and [^{3}H]-mazindol binding (black symbols) were studied *ex vivo* according to aforementioned protocols. These indices were quantified on suspensions obtained from striata injected with 300 pmol DEEP-NCS (columns), solvent (45% 2HγCD solution containing 0.5% DMSO: circles), and from striata opposite to DEEP-NCS injected side (triangles); Results are expressed as percentages of control rats treated with clomipramine. Data are means±s.e.mean of 4–8 experiments performed in duplicate.

One day after unilateral injections in rat striatum, DEEP-NCS produced similar dose-dependent decreases in both *ex vivo* indices of DAT availability. DEEP-NCS ($\geqslant$300 pmol) induced sub-maximal inhibitions (>90%) of the [^{3}H]-DA uptake and the [^{3}H]-mazindol binding (Table 4); ID_{50} values corresponded to 15 ± 0.95 pmol (i.e. 8 ng) and 13 ± 0.97 pmol (6.9 ng) DEEP-NCS striatum^{-1} for [^{3}H]-DA uptake and [^{3}H]-mazindol respectively.

DAT recovery after inactivation

A unilateral injection of 300 pmol DEEP-NCS (1 h after 16 mg kg^{-1} clomipramine i.p.) resulted in similar blockade and time course of recovery for *ex vivo* [^{3}H]-DA uptake and [^{3}H]-mazindol binding (Figure 3). The inhibition of uptake observed in injected striata one day after DEEP-NCS injection (95%) slowly decreased with time so that DA transport corresponded to 54 and 88% of that in control striata, 6 and 18 days after injection, respectively. The [^{3}H]-mazindol binding displayed a similar recovery profile. None of the indices of DAT availability was modified either in 2HγCD injected striata or in striata opposite to injections of DEEP-NCS (Figure 3) or solvent (not shown). The 15% inhibition of the *ex vivo* [^{3}H]-5-HT uptake generated by DEEP-NCS on day one, was reduced to an insignificant level ($\leqslant$5%) as early as 3 days after injection (Figure 3).

The inhibition of the *ex vivo* DA transport elicited by 300 pmol DEEP-NCS was due to a decrease in V_{max} and an increase in K_M (Figure 4). Very low values of uptake were obtained 1 day after DAT inactivation, impairing an accurate estimate of K_M and V_{max} values at this time. On day 3, the K_M was increased by 164% and the V_{max} was decreased by 36%. These changes disappeared according to similar time-courses (Figure 4), suggesting that the irreversible blockade of the DAT by DEEP-NCS partly resulted in a modification of the K_M value. Rate constants for degradation and recovery of the DAT were calculated from data presented in Figures 3 and 4. The mean rate constant for DAT degradation (k) was 0.115 day^{-1}, and the $t_{½}$ for DAT recovery was 6.1 days (Table 5). The transporter production rate (r) estimated from V_{max} recovery was approximately 12.2 pmol g striatum^{-1} day^{-1}.

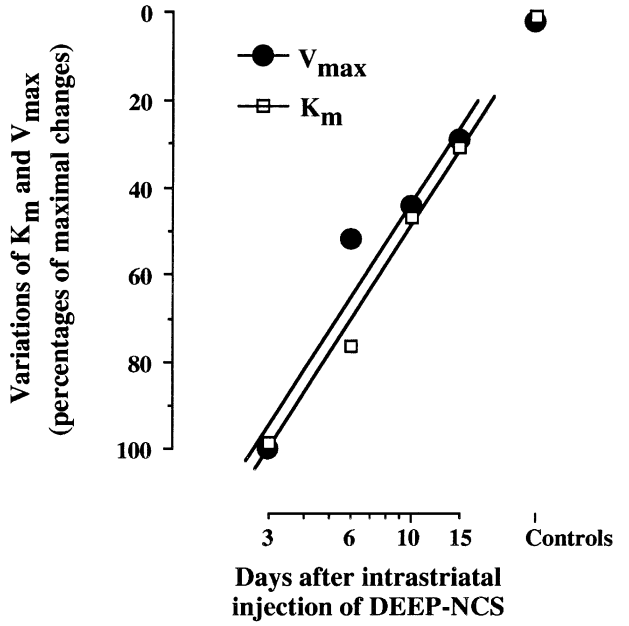

Figure 4 Time course (days) of the effect of an intrastriatal injection of 300 pmol DEEP-NCS on the kinetic constants of the neuronal uptake of [^{3}H]-DA measured *ex vivo*. Unilateral striatal injections (10 μl) were performed 1 h after pretreatment of animals with 16 mg kg^{-1} clomipramine (i.p). Rats were sacrificed and synaptosomal suspensions were prepared from striata injected with DEEP-NCS and striata from control rats treated with clomipramine. K_M (squares) and V_{max} (circles) for the [^{3}H]-DA uptake were determined *ex vivo* as described in Methods for saturation studies. Data are means$\pm$s.e.mean of four (DEEP-NCS)-six (solvent: 45% 2HγCD solution containing 0.5% DMSO) experiments performed in duplicate. Mean values for K_M and V_{max} were 281 nM and 267 pmol mg protein^{-1} min^{-1} respectively in solvent-injected striata and 812 nM and 180 pmol mg protein^{-1} min^{-1} respectively in DEEP-NCS injected striata at day 3.

mRNA levels after DAT inactivation

Stereotaxic injections of solvent (45% 2HγCD solution containing 0.5% DMSO), or DEEP-NCS (300 pmol) did not elicit any significant change in transporter mRNA level in SN ipsilateral to injected striata (Figure 5).

Discussion

We have characterized the ability of DEEP-NCS to irreversibly block the DAT, *in vitro* and *in vivo*, and we have used it to demonstrate that the low rate of DAT recovery reported elsewhere for the rat striatum (Fleckenstein *et al*., 1996) is likely to correspond to the physiological rate of DAT renewal.

DEEP-NCS displayed some features of an irreversible inhibitor *in vitro* and *in vivo*. First, its ability to block the DAT *in vitro* was inversely related to the protein concentration (Figure 2), a property which has already been described for other irreversible inhibitors of the DAT (Boja *et al*., 1990; 1991) and for agents alkylating peripheral benzodiazepine receptors and GABA A receptors (Lueddens *et al*., 1986; Lewin *et al*., 1989). Second, like other irreversible blockers of the DAT (Schweri *et al*., 1992; Zimanyi *et al*., 1989; Deutsch *et al*., 1992), DEEP-NCS produced a wash-resistant inhibition of the transporter, whereas the inhibition produced by two structurally related compounds of similar affinity, MR 14001 and MR 14503, or the reference inhibitor nomifensine, was totally reversed (Table 2). Third, the time-course for DAT recovery observed after an intrastriatal injection of DEEP-NCS seems to be markedly slower than that resulting from a systemic administration of a very potent and slowly-reversible GBR derivative (Rothman *et al*., 1991; Saadouni *et al*., 1994; Do-Régo *et al*., 1999). Thus, the $t_{½}$ for DAT recovery after DEEP-NCS injection was 5.3–6.9 days, whereas the $t_{½}$ for the

Table 5 Apparent constants for degradation and production of DAT after DEEP-NCS inactivation

	k (day^{-1})	$t_{½}$ (day)
K_M	0.10	6.9
V_{max}	0.11	6.3
DA uptake	0.13	5.3
Mazindol binding	0.12	5.8

Rate constant for DAT degradation (k) and $t2_{1/2}$ for DAT recovery were calculated from values presented in Figure 3 (DA uptake, mazindol binding) and Figure 4 (K_M, V_{max}), according to aforementioned equations. [*Tss*] was taken to be the concentration of recoverable parameter, i.e. the mean value of the parameter in controls minus residual value of this parameter for the first observation after DEEP-NCS inactivation.

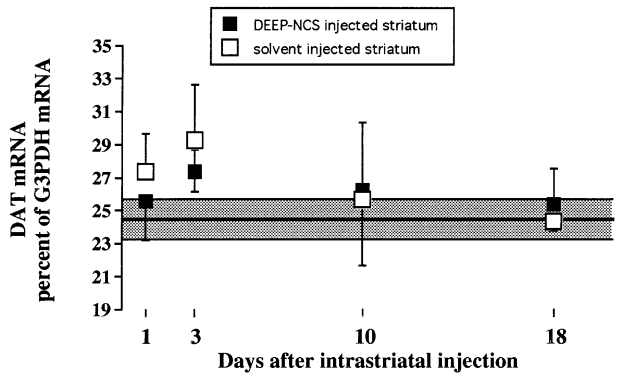

Figure 5 Time course of the effect of an intrastriatal injection of 300 pmol DEEP-NCS on mRNA level for DAT in SN. Unilateral striatal injections (10 μl) were performed 1 h after pretreatment of animals with 16 mg kg^{-1} clomipramine (i.p.). Rats were sacrificed and SN ipsilateral to striatum injected with solvent (45% 2HγCD solution containing 0.5% DMSO: open symbols), or DEEP-NCS (closed symbols) were dissected out. mRNA were amplified and quantified on extracts obtained from two SN and mRNA levels were expressed as percentages of mRNA for G3PDH. The shaded area corresponds to mean $\pm$ s.e.mean of mRNA level in SN from control rats treated with clomipramine. Data are means $\pm$ s.e.mean of 4–7 experiments (14 for controls). There was no significant change in transporter mRNA after intrastriatal injection of solvent ($t \leqslant 1.65$) or DEEP-NCS ($t \leqslant 1.15$).

clearance of GBR 12783 from the striatum was estimated to be in the 2 h range (Bonnet & Costentin, 1986; Chagraoui *et al*., 1987). In the same way, a single high dose of GBR 12909 (30 mg kg^{-1}) did not elicit any significant change in the rat striatal DAT availability, 1 day after its s.c. administration (Kunko *et al*., 1997).

DEEP-NCS produced an inhibition of the DA uptake which resulted from a decrease in V_{max} and an increase in K_M. The non-competitive component of transport blockade was expected and is consistent with properties of an irreversible inhibitor. On the contrary, the change in K_M is somewhat puzzling, more especially as it was also observed *ex vivo*. However, this situation is not exceptional. *In vitro*, metaphit produced a significant increase in dissociation constants for [^{3}H]-DA uptake and [^{3}H]-methylphenidate binding (Schweri *et al*., 1989), and the irreversible blockade of the peripheral benzodiazepine receptors and GABA A receptors resulted from mixed changes in K_D and B_{max} values (Lueddens *et al*., 1986; Lewin *et al*., 1989). Some studies performed *ex vivo* also give evidence that injection of an alkylating agent can increase the K_D value for binding to the target protein (Fleckenstein *et al*., 1996; Keck & Lakoski, 1996a, b). However, these increases were rather early (1–24 h after injection) and disappeared more quickly than decreases in B_{max}. Consequently, these K_D increases were probably due to the presence of residual free alkylating agent and/or to the formation of a first inhibitor-target protein complex which has not already evolved into alkylation.

These hypotheses are unlikely to explain the effects of DEEP-NCS on K_M values for [^{3}H]-DA uptake. As previously mentioned, the $t_{½}$ for the K_M value seems to be too high for it to correspond to the clearance of a slowly-dissociating inhibitor from the striatum. On the other hand, DEEP-NCS, like other GBR derivatives, could recognize other targets such as cytochrome P450IID1 or the piperazine acceptor site, but alkylation of proteins different from the DAT are unlikely to support changes in the kinetic constants of the DA uptake (Corera *et al*., 1998). On the contrary, the parallel return of K_M and V_{max} to control values observed in the present study (Figure 4), and the close similarity of the resulting $t_{½}$ values (Table 5) with that obtained after DAT inactivation by RTI-76 (Fleckenstein *et al*., 1996) strongly suggest that K_M and V_{max} changes originate from a common process, alkylation and renewal of the DAT. As a consequence, DEEP-NCS might recognize and alkylate two nucleophilic groups in the DAT: blockade of one of them may be responsible for the decrease in maximal transport activity whereas alkylation of the second one may produce a modification of the DAT structure resulting in an increased K_M. It is noteworthy that these nucleophilic groups can be located in the same binding domain. Alternatively, DEEP-NCS could also recognize different forms of the DAT, resulting in different alkylations and effects on both K_M and V_{max} of uptake (Coulter *et al*., 1995; Gracz & Madras, 1995; Jones *et al*., 1996).

The ability of DEEP-NCS to block the DAT *in vitro* and *in vivo* is difficult to compare with that of other irreversible inhibitors owing to the fact that experimental conditions, protein concentration (Figure 2) and solvents (Table 3) are critical for the estimate of this parameter. So, assuming that solvents did not drastically influence the inhibitor potencies, present results suggest that DEEP-NCS could be 50–100 times more potent than RTI-76 (ID_{50}: 1 nmol striatum^{-1} approx.) for blocking the DAT *in vivo* (Table 4). On the other hand, isothiocyanate derivatives of GBR 12783 have been reported to display *in vitro* an affinity for the DAT in the 0.1 μM range (Deutsch *et al*., 1992; Deutsch & Schweri, 1994).

Both *in vitro* and *ex vivo*, DEEP-NCS was devoid of any marked effect on the choline transporter which shares some structural homology with the DAT (Giros & Caron, 1993), demonstrating that it did not produce a non-specific alkylation of all proteins. However, *in vitro* experiments indicate that DEEP-NCS still recognizes neuronal transporters for NA and 5-HT, even if it keeps a moderate selectivity for the DAT (Table 1). *In vivo*, DEEP-NCS should not have any effect on NA transporters since they are located in cerebral structures other than striatum, and since DEEP-NCS solutions in 45% 2HγCD containing 0.5% DMSO diffuse only over a restricted area (Table 3, Figure 3). In the same way, experimental conditions chosen for studying DAT renewal rate almost totally protected 5-HT transporters from alkylation. Thus, 1 day after intrastriatal injection in rats pretreated by clomipramine, 300 pmol DEEP-NCS reduced indices of DAT availability by about 95% whereas 5-HT transport was impaired by 10–15% (Table 3, Figure 3). It is worthy to note that a pretreatment by clomipramine slightly decreased the *ex vivo* [^{3}H]-5-HT uptake in control preparations (Table 3).

Rate constants for degradation and recovery of the DAT estimated in the current work (Table 5) are fully consistent with those obtained with RTI-76, a 3β-phenyltropane derivative (Fleckenstein *et al*., 1996). The fact that these inhibitors are likely to differ from one another in their binding site on the DAT (Sallee *et al*., 1989; Patel *et al*., 1992; Vaughan *et al*., 1999) and in their pharmacological specificity did not influence the $t_{½}$ value. In both cases, it was estimated to be of 6 days. This rather long $t_{½}$ is also consistent with a recent work in which 7 days of intranigral infusion of antisense oligodeoxynucleotides targeting the DAT gene were required to observe a moderate decrease in striatal [^{3}H]-mazindol binding (Silvia *et al*., 1997). On the other hand, recovery of the DAT is probably not controlled at the transcription level since amounts of DAT mRNA in SN were not affected following transporter inactivation (Figure 5). Thus, the whole of these data support that turnover rate of the DAT in rat striatum *in vivo* could be actually of 6 days, even if one cannot

exclude that protein synthesis and post-translational events could constitute other regulation steps for DAT availability.

The rate of transporter production estimated after inactivation by DEEP-NCS (12.2 pmol g striatum^{-1} day^{-1}) seems lower than that reported following inactivation by RTI-76 (24 pmol g striatum^{-1} day^{-1}). In fact, this discrepancy comes from differences in DAT density in controls, since it was approximated to be 250 pmol g striatum^{-1} in the present work but 400 pmol g striatum^{-1} in the previous report (Fleckenstein *et al*., 1996).

Blockade of more than 50% of the DAT during 5 days did not provoke any change in its mRNA level. Alteration in mRNA levels seems by far harder to induce for DAT than for neurotransmitter receptors. A sub-total inactivatioan of 5-HT_{1A} receptors by EEDQ resulted in 74–364% increases in 5-HT_{1A} mRNA levels in different brain areas of the rat (Raghupathi *et al*., 1996b). These increases were early and rather long lasting (1–7 days). In the case of the DAT, repeated treatments with an uptake inhibitor only causes modest changes in its mRNA level. Thus, chronic cocaine administration to rats for 8 or 14 days produced small decreases (10–25%), or no change (Maggos *et al*., 1997) in mRNA level in ventral tegmental area (VTA) and/or SN compacta (Burchett & Bannon, 1997; Letchworth *et al*., 1997). On the contrary, no change (Persico *et al*., 1993) or moderate up-regulations (20–36%) of DAT mRNA were observed in both SN and VTA after 2–3 days of withdrawal from 5 days of amphetamine treatment (Lu & Wolf, 1997; Shilling *et al*., 1997). It is not clear why treatments which both increase dopaminergic transmission could lead to opposite changes, but one can suggest that the increase in the DAT-mediated uptake produced by amphetamine could result in an up-regulation of the gene expression whereas its blockade by cocaine could provoke the opposite regulation. At all events, previous reports and present data support that the transcriptional regulation of the DAT gene could be a very tightly controlled process.

In conclusion, DEEP-NCS displays *in vitro* several features of an irreversible inhibitor of the DAT. *In vivo*, it behaves as a potent, and at least somewhat selective, inhibitor of the DAT. The *in vivo* turnover rate of the transporter in rat striatum seems to be actually rather low, as suggested by a $t_{½}$ for DAT recovery of 6.1 days and the lack of regulation of DAT mRNA level in SN following inactivation by stereotaxic injection of DEEP-NCS. This irreversible inhibitor constitutes a useful tool for evaluating DAT renewal rate in different brain areas and during ontogeny or aging.

This work was supported in part by grants from D.R.E.T. (contract 95–167). The authors gratefully acknowledge the excellent and friendly technical advice of M. Daoust and M. Naassila for mRNA quantification and Dr A.-R. Schoofs (C.E.B.) for managing the DEEP-NCS synthesis.

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(Received February 1, 1999
Revised June 7, 1999
Accepted June 18, 1999)