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Binding of Pinoline on the 5-Hydroxytryptamine Transporter: Competitive Interaction with [^{3}H] Citalopram

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Abstract: Pinoline (6-methoxy-1,2,3,4-tetrahydro-β-carboline) is a naturally occurring compound in the mammalian body which inhibits 5-hydroxytryptamine (5-HT) uptake and exerts antidepressant-like behavioural effects in rats. The present study investigates the effects of pinoline on [^{3}H]citalopram binding to the 5-HT transporter on rat brain. Our experiments revealed that pinoline inhibits [^{3}H]citalopram binding with IC_{50} 1255±167 nM and K_i 572±76 nM; Hill coefficient for inhibition was close to 1. In saturation experiments, pinoline co-incubated with [^{3}H]citalopram, increased dose-dependently the K_d value but had no effect on the B_{max} value of [^{3}H]citalopram binding. Micromolar concentrations of pinoline did not have influence on the dissociation rate of specifically bound [^{3}H]citalopram. Binding parameters of [^{3}H]citalopram did not differ significantly in cerebral cortex and hippocampus of rats treated for 10 days with pinoline or vehicle. These results indicate that pinoline did not have any modulative influence on the activity of 5-HT transporter and it interacts competitively with citalopram on the substrate recognition site of the 5-HT transporter.

ß-Carbolines have been in the focus of interest since the McIsaac's discovery that these compounds may form in the mammalian body under physiological conditions (McIsaac 1961). Among endogenously found β-carbolines probably most attention has been given to the pinoline (6-methoxy-1,2,3,4-tetrahydro-β-carboline, 6-MeO-THBC, 5-methoxytryptoline). This compound has been found in pineal and adrenal glands, in retina and in brain in concentrations ranging from 2 ng/g or below that (Barker *et al.* 1981; Langer *et al.* 1986), 0.1–1 μg/g (Leino *et al.* 1983; Kari *et al.* 1983) up to concentration 21 μg/g (Langer *et al.* 1984b). In rat and human blood the concentration of endogenous pinoline has been found below the detection limit, although pinoline has been found in trace amounts in human platelets (Schouten & Bruinvels 1985).

Pinoline inhibits 5-hydroxytryptamine (serotonin, 5-HT) uptake and displaces efficiently tritiated imipramine from its binding sites in brain (Komulainen *et al.* 1980) and platelets (Segonzac *et al.* 1985). Initially pinoline was proposed to modulate the activity of serotonin transporter by acting as an endogenous ligand of the [^{3}H]imipramine recognition site (Langer *et al.* 1984a & b; Segonzac *et al.* 1985). Now it is widely recognised that both imipramine and 5-HT bind to the same site in serotonin transporter (Marcusson & Ross 1990). Therefore, the endogenous ligand for imipramine binding site is considered to be serotonin and investigations for additional endogenous ligands are subsided. However, it is supposed that the serotonin transporter may have an additional regulatory binding site. It has been shown that 5-HT and some antidepressants at high concentrations may slow the dissociation of high-affinity bound 5-HT transporter ligands (Plenge & Mellerup 1985). The effect of pinoline on the dissociation of specifically bound 5–HT transporter ligands in brain tissue has not been studied before.

Recently we demonstrated that after acute administration, pinoline has a clear and dose-dependent antidepressant-like effect in rats forced swimming test. Moreover, the effects of pinoline in the elevated plus-maze test resembled those of serotoninergic drugs (Pähkla *et al.* 1996). The aim of the present study was to elucidate possible neurochemical mechanisms of the behavioural effects of pinoline. The experiments were designed to study the interaction of pinoline with serotonin transporter by using competition with [^{3}H]citalopram which is the most selective ligand for 5-HT transporter available at present. To reveal possible interaction with proposed allosteric binding site in serotonin transporter the influence of pinoline on the dissociation kinetics of [^{3}H]citalopram brain membrane complexes was investigated and compared with that of antidepressants. To assess the possible influence of chronic pinoline treatment on the affinity and number of binding sites of serotonin transporter the [^{3}H]citalopram binding parameters were determined in the cerebral cortex and hippocampus of rats treated for 10 days with pinoline.

Materials and Methods

Materials. Male Wistar rats ("Grindex" breeding laboratory, Riga, Latvia) weighing 230–350 g were used for all experiments. [^{3}H] Citalopram (80.0–81.4 Ci/mmol) was obtained from NEN (Boston, MA, U.S.A.). The following drugs were obtained from their pharmaceutical company of origin or regular commercial sources: citalo-

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pram hydrobromide (H. Lundbeck AS, Copenhagen, Denmark), fluoxetine hydrochloride (Eli Lilly and Co, Indianapolis, IN, U.S.A.), harman (1-methyl-β-carboline) (Fluka AG, Buchs, Switzerland), DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate) (Schering AG, Berlin, Germany), imipramine hydrochloride, desipramine hydrochloride, 5-HT creatinin sulfate, tryptamine, melatonin, pinoline, noreleagnine (1, 2, 3, 4-tetrahydro-β-carboline), harmalol (7-hydroxy-1-methyl-dihydro-β-carboline) hydrochloride (all from Sigma, St. Louis, MO, U.S.A.). All other reagents were standard laboratory reagents of analytical grade.

Brain membrane preparation. Rats were decapitated, the brains removed, whole cerebral cortex from all rats and hippocampi from chronically treated animals were dissected on an ice-cooled glass plate and stored at −70° until use. Crude synaptic membranes were prepared according to Plenge *et al.* (1990) with slight modifications (instead of fresh tissues, frozen tissues were used and the pH of buffers was 7.4 instead of 7.5). In brief, tissues were homogenised for 10 sec. with Kinematica Polytron homogeniser (80% maximal speed) in 1:30 w/v of ice-cold buffer (50 mM Tris-HCl, 150 mM NaCl, 20 mM EDTA; pH 7.4) and centrifuged at 30.000 ×g for 15 min. at 4°. Membranes were lysed for 30 min. at 0° and rehomogenised for 5 sec. in 10 ml of buffer (5 mM Tris-HCl, 5 mM EDTA; pH 7.4). Membranes were collected by centrifugation for 15 min. at 30.000 ×g. The resulting pellet was washed three times with 12 ml of incubation buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl; pH 7.4) by centrifugation at 30.000 ×g for 15 min. at 4°. The final pellet was suspended to a concentration of 0.5–0.6 mg protein/ml in the binding assay for saturation and competition experiments and 1 mg protein/ml for dissociation experiments. Protein concentration in the assay was measured according to Lowry *et al.* (1951).

Inhibition of [^{3}H]citalopram binding by various drugs. Rat brain membranes were incubated with 1.4 nM [^{3}H] citalopram in the presence of 8–12 concentrations of unlabelled drug for 60 min. at 22° in a final volume of 250 µl. Triplicate determinations were used in each experiment. Incubations were terminated by the addition of 3 ml of ice-cold buffer and membranes were collected by filtration under vacuum onto Whatman GF-B glass fibre filters. Filters were washed with two consecutive 3 ml aliquots of ice-cold buffer, transferred into scintillation vials containing 4 ml of scintillation fluid (Hi-Safe, Wallac) and stored at room temperature for 24 hr. Radioactivity trapped by the filters was determined by liquid scintillation spectrometry. K_i values were calculated using the equation $K_i = IC_{50}/(1+C:K_d)$, where IC_{50} is the concentration at which the compound displaces 50% of the specifically bound labelled ligand, C is the concentration of labelled ligand and K_d is the equilibrium dissociation constant (Cheng & Prusoff 1973). IC_{50} values were determined by non-linear variable slope analysis with multipurpose data analysing program Prism 2.0 (GraphPad Software Inc., San Diego, CA, U.S.A.). Hill coefficients were determined from Hill plots of saturation experiments with curve-fitting computer program Enzfitter (Leatherbarrow, 1987).

Equilibrium saturation studies. B_{max} and K_d for [^{3}H]citalopram binding were determined by 60 min. incubations at 22°. The reaction mixture contained 175 µl tissue suspension, [^{3}H]citalopram at one of seven concentrations between 0.12 and 21.0 nM and a displacer for determining non-specific binding or a β-carboline derivative in a total volume of 250 µl. The specific binding was defined as the difference between the total binding and that remaining in the presence of 10 µM fluoxetine. In the Scatchard analysis pinoline and noreleagnine were used at concentrations close to their IC_{30} and IC_{60} values (0.4 µM and 1.5 µM for pinoline and 4µM and 15 µM for noreleagnine respectively). Saturation curves of radioligand binding were analysed using the non-linear least squares regression with curve fitting program Enzfitter (Leatherbarrow 1987).

Dissociation rate determinations. Rat brain membranes were incubated with 5 nM [^{3}H]citalopram at 22° for 60 min. The dissociation was initiated by diluting 200 µl aliquots of membrane suspension into 10 ml buffer (50xdilution) at 22° containing the various drugs. The samples were filtrated through Whatman GF-B glass-fibre filters immediately after the dilution and at different dissociation times up to 240 min. after the 50× dilution step. Filters were washed thereafter with 2×5 ml ice cold buffer. To determine the non-specific binding, tubes from each dissociation rate determination were heated to 37° for 3 hr, ensuring that all specifically bound ligand had dissociated from the binding site. The samples were then returned to 22° for 1 hr before filtration. The radioactivity was counted as described above. As the [^{3}H]citalopram receptor-ligand complex dissociates according to first-order kinetics (Bennet & Yamamura 1985), the half-life ($t_{1/2}$) in minutes of complexes was determined from the first order equation $B = B_0 e^{-kt}$, from which $t_{1/2} = \ln 2/k_{-1}$. The dissociation rate constant (k_{-1}) was obtained by non-linear regression using the one phase exponential decay equation with data analysing program Prism 2.0.

Subchronic treatment of rats. Rats were injected daily at 10–12 a.m. intraperitoneally with 10 mg/kg of pinoline for 10 days. Pinoline was suspended in aqueus suspension of Tween 85 (3% vol.). To control group a Tween 85 solution of the same concentration as a vehicle was administered. Rats were killed 24 or 48 hr after the last injection. The significance of difference between binding results after sub-chronic treatments was calculated with Student's t-test.

Results

Our experiments revealed that [^{3}H] citalopram binds in rat cortical membranes to the single population of binding sites with a K_d of 1.56±0.21 nM and a B_{max} of 420±40 fmol/mg protein. About 85% of the total binding was displaceable. All antidepressants studied inhibited the specific [^{3}H] citalopram binding in a monophasic pattern with Hill coefficients

Table 1.

Inhibition of [3H]citalopram binding to rat cortical membranes by some antidepressants, tryptamines and ß-carbolines.

Drug	n	K_i (nM)	nH
Citalopram	4	0.75±0.16	1.04±0.08
Fluoxetine	3	25.9±3.2	1.16±0.15
Imipramine	3	65.2±6.2	1.07±0.10
Desipramine	3	347±27	1.36±0.26
5-HT	3	301±39	1.13±0.09
Tryptamine	2	3600±420	0.87±0.11
Melatonin	2	300 000±80 000	–
Pinoline	4	572±76	0.96±0.04
Noreleagnine	3	7032±984	1.04±0.06
Harmalol	2	14 800±3 800	0.78±0.14
Harman	2	19 600±4 400	0.76±0.15
DMCM	2	>1mM	–

The inhibition of [^{3}H]citalopram binding was determined at 1.3–1.5 nM [^{3}H]citalopram for the various compounds listed. Eight to twelve concentrations of displacer were used for each determination. The concentration of displacer that inhibited specific binding by 50% (IC_{50} value) was determined from the data using computer-assisted curve fitting with GraphPad Prism 2.0 program. K_i values for each displacer were subsequently calculated from IC_{50} value. Hill coefficients (nH) were determined from Hill plots of saturation experiments. The values shown in the table represent mean±S.E.M. of 2–4 determinations done in triplacte; n is the number of experiments with each compound.

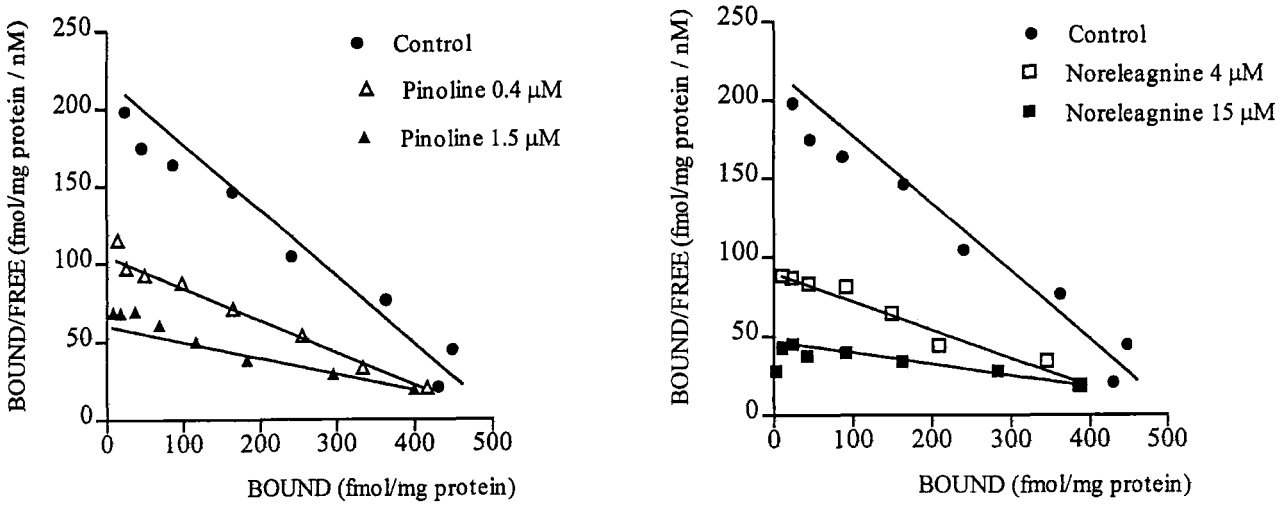

Fig. 1. Influence of pinoline (A) and noreleagnine (B) on Scatchard plots of [^{3}H]citalopram binding. Crude membrane homogenates were incubated with [^{3}H]citalopram (0.12–20.6 nM) in triplicate for 60 min. at 22° in the presence of 0.4 μM or 1.5 μM pinoline; 4 μM or 15 μM noreleagnine; or in the absence of other drugs (control). The data from typical experiments shown are: in the absence of added drug K_d 2.34±0.40 nM, B_{max} 515±24 fmol/mg protein; in the presence of 0.4 μM pinoline K_d 4.88±0.21 nM, B_{max} 510±8 fmol/mg protein; in the presence of 1.5 μM pinoline K_d 9.78±0.69 nM, B_{max} 586±19 fmol/mg protein; in the presence of 4 μM noreleagnine K_d 5.62±0.75 nM, B_{max} 505±27 fmol/mg protein; in the presence of 15 μM noreleagnine K_d 14.79±1.03 nM, B_{max} 618±25 fmol/mg protein. Three different experiments were performed with each compound with similar results.

near unity (table 1). The most potent inhibitor was unlabelled citalopram with IC_{50} 1.53±0.31 nM and K_i 0.75±0.16 nM, the least potent was desipramine with IC_{50} 747±58 nM and K_i 347±27 nM.

Like antidepressants, 5-HT also seems to inhibit [^{3}H]citalopram binding competitively. The K_i value 0.3 mM for melatonin is only approximate because melatonin did not inhibit specific [^{3}H]citalopram binding completely up to the concentration of 1 mM (the highest concentration of displacing agent used in our experiments).

All β-carbolines studied were weaker inhibitors of [^{3}H]citalopram binding than 5-HT or antidepressants. The inhibition appeared to be monophasic and gave Hill coefficients close to 1. The most potent β-carboline compound was pinoline with IC_{50} 1255±167 nM and K_i 572±76 nM. Benzodiazepine receptor inverse agonist DMCM showed very little activity to displace [^{3}H] citalopram from its binding site and gave IC_{50} values greater than 1mM.

In saturation experiments a Scatchard plot carried out in the presence of pinoline or noreleagnine was linear but not parallel with that performed in the absence of added drug (fig. 1). Co-incubation with 0.4 μM or 1.5 μM of pinoline and 4 μM or 15 μM of noreleagnine did not change the B_{max} value. However, the affinity of [^{3}H]citalopram binding was significantly and dose-dependently decreased in the presence of both compounds.

Fig. 2 shows the effect of a 50× dilution with the buffer alone or with buffer containing different drugs at micromolar concentrations. The dissociation of [^{3}H]citalopram brain membrane binding site complex induced by dilution followed a monoexponential curve with half-life of 55.4 min. Fluoxetine increased the dissociation rate of receptor-ligand complex to half-life of 38.3 min. citalopram and imipramine decreased the dissociation rate ($t_{1/2}$ 353.6 min. for 200 μM citalopram and 64.6 min. for 200 μM imipramine). Pinoline did not have significant influence on the dissociation rate of receptor-ligand complex and both concentrations tested had $t_{1/2}$ values close to those with dilution with the buffer alone ($t_{1/2}$ 52.9 min. for 100 μM pinoline and 60.3 min. for 200 μM pinoline, respectively).

The K_d and B_{max} values of [^{3}H]citalopram binding in cerebral cortex and hippocampus of rats treated subchronically with pinoline or vehicle are presented in table 2. Student's t-test revealed no significant difference in these values between treatment groups in either brain structure. Difference between treatment with pinoline or vehicle was not significant in either time intervals after last injection studied (24 and 48 hr, respectively).

Discussion

[^{3}H]imipramine has been used extensively in binding assays to label the serotonin uptake transporter site and to investigate interactions of β-carbolines with this transporter (Langer *et al.* 1984a & b; Segonzac *et al.* 1985; Airaksinen & Kari 1991). However, most of former studies did not take into consideration the heterogeneity of imipramine binding. Only about 50% of desipramine defined specific [^{3}H]imipramine binding involves serotonin uptake sites (D'Amato *et al.* 1987). [^{3}H]Citalopram, in contrast, labels selectively serotonin uptake sites (Hyttel 1982). Therefore, we used [^{3}H]citalopram to characterise the interactions of β-carbolines with serotonin transporter.

The results of present experiments confirmed the findings of earlier studies with [^{3}H]imipramine that pinoline and noreleagnine competitively inhibit the high affinity binding of

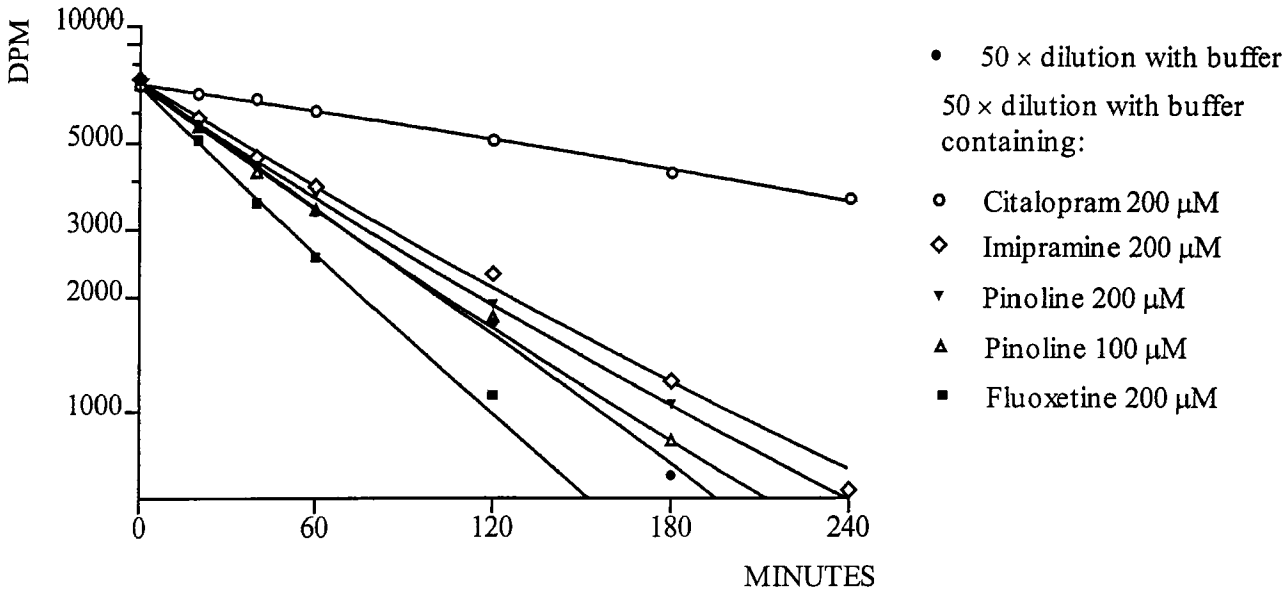

Fig. 2. [^{3}H]Citalopram dissociation from rat brain membranes at 22°. Crude membrane homogenates (200 μl) were incubated with 5 nM [^{3}H]citalopram in triplicate and diluted 50× into 10 ml buffer alone or into buffer containing 200 μM fluoxetine, 100 μM pinoline, 200 μM pinoline, 200 μM imipramine or 200 μM citalopram. Ordinate: d.p.m. in sample in logarithmic scale. Abscissa: time in min. after start of the experiment by a 50× dilution. Experiment was repeated twice with similar results.

serotonin transporter ligands. From β-carboline compounds studied pinoline was the most potent compound to inhibit [^{3}H]citalopram binding. Hill coefficients for pinoline and noreleagnine were not significantly different from unity as it is shown for antidepressants having 5-HT uptake inhibiting properties. Unsaturated β-carbolines harmalol and harman which had very low affinity on the [^{3}H]citalopram binding site showed shallow competition curves and Hill coefficients below 1. This may show noncompetitive inhibition and most probably, the low Hill coefficient is a result of non-specific interactions of receptor protein with displacer at very high concentrations.

Table 2.

Comparison of [^{3}H]citalopram binding parameters in the cerebral cortex and hippocampus of rats treated 10 days with vehicle or pinoline.

Time after last injection (hr)	Treatment	n	K_d±S.E.M. (nM)	B_{max}±S.E.M. (fmol/mg protein)
Cerebral cortex				
24	Control	5	1.52±0.07	489±20
	Pinoline	4	1.43±0.25	507±33
48	Control	5	1.37±0.10	591±21
	Pinoline	4	1.50±0.15	571±20
Hippocampus				
24	Control	5	1.57±0.12	488±18
	Pinoline	4	1.61±0.10	511±19
48	Control	5	1.40±0.08	456±19
	Pinoline	4	1.45±0.14	454±31

Rats were treated intraperitoneally with pinoline 10 mg/kg in aqueous suspension of Tween 85 or with Tween 85 solution of the same concentration (control) for 10 days. Crude membrane homogenates were incubated for 60 min. at 22° with increasing concentrations of [^{3}H]citalopram (0.15–18.00 nM) in duplicate; n is the number of rats in each group.

A further confirmation of a competitive interaction was obtained from saturation studies. Both pinoline and noreleagnine decreased the affinity but had no influence on the number of binding sites for [^{3}H]citalopram as it is characteristic to the competition for the same binding site.

The dissociation experiments revealed that pinoline did not have any influence on the dissociation rate of high-affinity bound [^{3}H]citalopram. In former studies it has been shown that citalopram has the greatest slowing effect on the dissociation rate of labelled serotonin transporter ligand and receptor complexes; dissociation is considerably slowed also in the presence of serotonin (Plenge & Mellerup 1985, Wennogle & Meyerson 1985; Plenge *et al.* 1990). The reason of this effect is not known but it is suggested that serotonin transporter may have an allosteric binding site, the activation of which locks the transporter in a configuration in which dissociation of drugs bound to the binding site is inhibited (Plenge *et al.* 1990). It is suggested that there may exist an endogenous modulator which regulates the serotonin uptake activity and diurnal variation in the 5-HT uptake (Wennogle & Meyerson 1985; Rovescally *et al.* 1989). Although it has been reported that endogenous levels of pinoline in chicken fluctuate in a similar way to those of melatonin (Kari *et al.* 1983) our study revealed that pinoline does not have any influence to the site modulating the dissociation of bound [^{3}H]citalopram. The tissue concentration of pinoline was not determined in the present study. However, the possible influence of endogenous pinoline to the results of experiments was minimised by hypoosmotic

lysis and extensive washing of tissues during the membrane preparation procedure.

The absence of any influence to the affinity and number of binding sites for [^{3}H]citalopram was further supported by determination of [^{3}H]citalopram binding characteristics after subchronic treatment of rats with pinoline. This effect was similar to that observed mostly after chronic treatment of rats with tricyclic antidepressants or 5-HT uptake inhibitors (for review see Marcusson & Ross 1990).

In conclusion, our study revealed that pinoline did not have any modulative influence on the activity of serotonin transporter and it interacts competitively with 5-HT uptake inhibitors to the substrate recognition site of the transporter. In addition, pinoline is reported to inhibit monoamine oxidase A activity (Glover *et al.* 1982; Fernandez de Arriba *et al.* 1994), but this effect appears in much higher drug concentrations. Although the inhibition of monoamine oxidase A may take part in some effects of pinoline, the antidepressant-like and serotoninergic effects in behavioural experiments on rats (Pähkla *et al.* 1996) are probably mainly the result of serotonin reuptake inhibition. Considering the comparatively low endogenous levels of pinoline and competitive interaction with serotonin, our study does not support the role of pinoline as endogenous modulator of the activity of serotonin transporter in the rat brain.

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