Br. J. Pharmacol. (1993), **108**, 597–603

Chronic treatment with the monoamine oxidase inhibitors clorgyline and pargyline down-regulates non-adrenoceptor [^{3}H]-idazoxan binding sites in the rat brain

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1 The binding of [^{3}H]-idazoxan in the presence of 10^{-6} M (−)-adrenaline was used to quantitate non-adrenoceptor idazoxan binding sites (NAIBS) in the rat brain after treatment with various psychotropic drugs.

2 Chronic treatment (14 days) with the monoamine oxidase (MAO) inhibitors clorgyline (0.3–10 mg kg^{-1}, i.p.) and pargyline (10 mg kg^{-1}, i.p.), but not with Ro 41-1049 (1 mg kg^{-1}, i.p.), markedly decreased (30–50%) the density of NAIBS in the cerebral cortex without any apparent change in the affinity of the radioligand.

3 Acute (1 day) and/or chronic treatments (14 days) with other psychotropic drugs such as desipramine (3 mg kg^{-1}, i.p.), cocaine (10 mg kg^{-1}, i.p.), reserpine (0.12 mg kg^{-1}, s.c.), haloperidol (1 mg kg^{-1}, i.p.) and diazepam (10 mg kg^{-1}, i.p.) did not alter the density of NAIBS in the cerebral cortex.

4 *In vitro*, the propargylamines clorgyline, pargyline and deprenyl displaced the binding of [^{3}H]-idazoxan to NAIBS from two distinct sites, but only clorgyline displayed an apparent very high affinity for a relevant population of NAIBS (K_{iH} = 40 pM; K_{iL} = 10.6 μM). The structurally diverse MAO inhibitors Ro 16-6491 (selective for MAO-B) and Ro 41-1049 (selective for MAO-A), as well as the other psychotropic drugs (desipramine, cocaine, reserpine and haloperidol) displaced the binding of [^{3}H]-idazoxan to NAIBS monophasically and with very low potencies. As expected, the MAO inhibitors clorgyline and Ro 41-1049 displaced the binding of [^{3}H]-Ro 41-1049 to MAO-A monophasically and with high potencies (K_i values: 0.18 nM and 22 nM, respectively). In contrast, idazoxan displayed very low affinity (K_i = 40 μM) against the binding of [^{3}H]-Ro 41-1049 to MAO-A. These results disprove a direct interaction between [^{3}H]-idazoxan and the enzyme MAO.

5 Preincubation of cortical membranes with clorgyline (10^{-9} M or 10^{-6} M for 30 min) or pargyline (10^{-6} M or 10^{-5} M for 30 min), reduced by 30–50% and by 17–30%, respectively, the total density of NAIBS without any apparent change in the affinity of the radioligand. Preincubation with 10^{-6} M clorgyline did not alter the affinity of cirazoline for the two populations of NAIBS, but reduced by 60% the binding of [^{3}H]-idazoxan to the high affinity site without affecting the binding of the radioligand to the low affinity site. These results indicate that the two MAO inhibitors irreversibly block the binding of [^{3}H]-idazoxan to NAIBS.

6 *In vivo*, however, various acute treatments with clorgyline (1–20 mg kg^{-1}, i.p.) for different time intervals (6–48 h) did not alter the density of NAIBS. *In vivo*, only very high doses of clorgyline (40 and 80 mg kg^{-1}, i.p.) induced modest decreases (21–28%) in the density of NAIBS in the cerebral cortex.

7 Together the results indicate that the irreversible binding of clorgyline and pargyline to NAIBS found *in vitro* does not fully explain the marked decreases in the density of NAIBS found *in vivo* after the chronic treatments. It is suggested that the down-regulation of NAIBS induced *in vivo* by clorgyline and pargyline, through a direct or indirect mechanism, may have functional implications.

Keywords: Non-adrenoceptor idazoxan binding sites; NAIBS; [^{3}H]-idazoxan; rat brain; MAO inhibitors; psychotropic drugs

Introduction

Evidence has accumulated that imidazoli(di)ne compounds such as idazoxan and *p*-aminoclonidine bind with high affinity not only to α_2-adrenoceptors, but also to non-adrenoceptor sites in a variety of species and tissues, including the brain (for a review see Michel & Insel, 1989; Atlas, 1991; Kilpatrick *et al.*, 1992). Non-adrenoceptor [^{3}H]-idazoxan binding sites (NAIBS) appear to be pharmacologically different from those labelled by [^{3}H]-*p*-aminoclonidine (Michel & Insel, 1989; Hieble & Ruffolo, 1992; Ernsberger, 1992) and also differ from α_2-adrenoceptors in in their biochemical properties and anatomical distribution (Boyajian *et al.*, 1987; Mallard *et al.*, 1992). Additional distinction between NAIBS and α_2-adrenoceptors refers to the subcellular localization of NAIBS on the mitochondrial outer membrane (Tesson & Parini, 1991; Tesson *et al.*, 1991) as well as on a different position of the cell surface in some tissues (Diamant *et al.*, 1992).

Endogenous depression appears to be associated with increased brain α_2-adrenoceptors ([^{3}H]-clonidine and [^{3}H]-UK 14304 binding) and down-regulation of these inhibitory receptors has been involved in the mechanism of action of various antidepressant drugs (Meana *et al.*, 1992 and other references therein). Recently, NAIBS as well as non-adrenoceptor binding sites labelled by [^{3}H]-*p*-aminoclonidine have also been found increased in postmortem brains of depressed suicide victims (Barturen *et al.*, 1992 and unpub-

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lished results) and in platelet membranes from depressed patients (Piletz et al., 1990; 1991). These imidazoline-preferring receptors also appear to mediate a presynaptic inhibition on the release of noradrenaline (NA) in adrenergic nerve terminals (Göthert & Molderings, 1991; Molderings et al., 1991). Therefore, enhanced imidazoline-preferring receptor (and α_2-adrenoceptor) activity and with it decreased NA release could be involved in the pathogenesis of endogenous depression.

In this context, the present study was designed to assess the effects of chronic antidepressant drugs (clorgyline and other monoamine oxidase (MAO) inhibitors, and desipramine) and other psychotropic drug treatments on NAIBS and also to investigate *in vitro* the pharmacological properties of the interaction of these drugs with the imidazoline binding sites.

Methods

Animals and treatments

Male Sprague-Dawley rats (250–300 g) were used. The animals received a standard diet with water freely available and were housed at 20 ± 2°C with a 12 h light/dark cycle. In the chronic (14 days) treatments the animals received i.p. every 12 h either 0.9% saline vehicle, the MAO-A inhibitors clorgyline (0.3–10 mg kg^{-1}) or Ro 41-1049 (1 mg kg^{-1}), the MAO-B inhibitor pargyline (10 mg kg^{-1}), and the NA re-uptake blockers desipramine (3 mg kg^{-1}) or cocaine (10 mg kg^{-1}). Rats were also treated with reserpine (0.12 mg kg^{-1} s.c., every 48 h for 14 days) to assess the effect of depletion of NA on NAIBS. In another series of experiments, rats were treated i.p. every 12 h for 14 days with haloperidol (1 mg kg^{-1}) or diazepam (10 mg kg^{-1}) for comparison. In the acute (1 day) treatments, the rats received two doses (12 h apart) of clorgyline (1 mg kg^{-1}) or desipramine (3 mg kg^{-1}). Both in the acute and chronic treatments the rats were killed 48 h after the last injection. In some experiments, the acute effects of various doses of clorgyline (10–80 mg kg^{-1}) on NAIBS were assessed at different time intervals (6, 12, 24 and 48 h).

Preparation of membranes

Neural membranes (P_2 fractions) were prepared by established methods (Giralt & García-Sevilla, 1989) from the parietooccipital cortex (frozen). Briefly, the tissue samples were homogenized in 5 ml of ice-cold Tris-sucrose buffer (5 mM Tris-HCl; 250 mM sucrose; 1 mM $MgCl_2$; pH 7.4). The homogenates were centrifuged at 1,100 *g* for 10 min, and the supernatants were then recentrifuged at 40,000 *g* for 10 min. The resulting pellet was washed twice with 2 ml of fresh incubation buffer (50 mM Tris-HCl, 0.1% ascorbic acid, pH 7.5) for [^{3}H]-idazoxan binding assays and 50 mM Tris-HCl, 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, pH 7.4 for [^{3}H]-Ro 41-1049 binding assays (Cesura *et al.*, 1990a). The final pellet was resuspended in an appropriate volume of this buffer to a final protein content of 800–1000 μg ml^{-1}. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

[^{3}H]-idazoxan binding assay

Total [^{3}H]-idazoxan binding was measured in 1.1 ml-aliquots (50 mM Tris-HCl, 0.1% ascorbic acid, pH 7.5) of the neural membranes which were incubated with shaking for 30 min at 25°C. Binding of [^{3}H]-idazoxan to NAIBS was always done in the presence of 10^{-6} M (−)-adrenaline to prevent the binding of the radioligand to α_2-adrenoceptors. Nonspecific binding was determined in the presence of 10^{-4} M naphazoline, as previously described (Olmos *et al.*, 1992). In the saturation studies, cortical membranes were incubated with eight concentrations of [^{3}H]-idazoxan (6×10^{-10} M to 5×10^{-8} M) as above. Specific binding (97% to 30%) was defined as the difference between total non-adrenoceptor binding (in the presence of 10^{-6} M adrenaline) and nonspecific binding (determined with 10^{-4} M naphazoline) and was plotted as a function of increasing concentrations of the radioligand. In the drug competition studies, cortical membranes were incubated as above with [^{3}H]-idazoxan (4×10^{-9} M, in the presence of 10^{-6} M adrenaline) and in the absence or presence of various concentrations of the competing drugs (10^{-14} M to 10^{-3} M; 15–24 concentrations). Total binding was determined as above and plotted as a function of the drug concentration.

In some experiments, the effects of *in vitro* preincubation with clorgyline or pargyline on [^{3}H]-idazoxan binding to NAIBS were tested. The pellet resulting from the last centrifugation (see preparation of membranes) was resuspended in 10 ml of fresh incubation buffer and incubated for 30 min at 25°C in the presence of two concentrations of clorgyline (10^{-9} M and 10^{-6} M) or pargyline (10^{-6} M and 10^{-5} M). Then neural membranes were washed twice with 10 ml of fresh incubation buffer and the final pellet was resuspended as above and used in the saturation and competition binding experiments.

Incubations were terminated by diluting the samples with 5 ml of ice-cold Tris incubation buffer (4°C). Membrane-bound [^{3}H]-idazoxan was measured by vacuum filtration, using a Brandel 48R cell harvester (Biomedical Research & Development Laboratories, U.S.A.), through Whatman GF/C glass fibre filters, which had been presoaked with 0.5% polyethylenimine (Bruns *et al.*, 1983). Then the filters were rinsed twice with 5 ml of incubation buffer, air-dried, transferred to minivials containing 5 ml of OptiPhase 'HiSafe' II cocktail (LKB, England) and counted for radioactivity by liquid scintillation spectrometry at 50% efficiency (Packard model 300 C).

[^{3}H]-Ro 41-1049 binding assay

Binding of [^{3}H]-Ro 41-1049 was performed as previously described (Cesura *et al.*, 1990a) with modifications (e.g. bound/free radioligand was separated by filtration instead of centrifugation). These assays were similar to those described above for [^{3}H]-idazoxan except in the buffer composition (50 mM Tris-HCl, 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, pH 7.4) and in the incubation conditions (60 min at 37°C). Drug competition studies were performed as above with [^{3}H]-Ro 41-1049 (4.5×10^{-9} M) in the absence or presence of various concentrations of the competing drugs (10^{-14} M to 10^{-2} M; 21 concentrations). The specific binding of [^{3}H]-Ro 41-1049, defined with 10^{-6} M clorgyline, represented about 98% of total binding.

Analyses of binding data and statistics

Analyses of saturation isotherms (K_d, dissociation constant; B_{max}, maximum density of binding sites) and competition experiments (K_i, inhibition constant) as well as the fitting of data to the appropriate binding models were performed by computer-assisted nonlinear regression using the EBDA-LIGAND (Munson & Rodbard, 1980; McPherson, 1985) or GraFit (Leatherbarrow, 1990) programmes. All experiments were initially analysed assuming a one-site model of radioligand binding and then assuming a two-site binding model. The selection between the different binding models was made statistically by the extra sum of squares principle (F test) as outlined by Munson & Rodbard (1980). The more complex model was accepted if the P value resulting from the F test was less than 0.05. For other details of the computer analyses see Olmos *et al.* (1992).

Results are expressed as mean ± s.e.mean. One-way analysis of variance (ANOVA), followed by Scheffé's test, was

used for the statistical evaluations. The level of significance was $P = 0.05$.

Drugs

[^{3}H]-idazoxan (specific activity, 40–48 Ci mmol^{-1}) was purchased from Amersham International plc (U.K.). [^{3}H]-Ro 41-1049 (specific activity, 30.8 Ci mmol^{-1}), was a generous gift from Dr J.G. Richards and Dr J. Saura (F. Hoffmann-La Roche Ltd., Switzerland). Both radioligands were stored at − 30°C. For the binding assays, appropriate amounts of the stock solutions were diluted with distilled and purified water (Milli-Q) containing 2.5 mM HCl and 6% ethanol. Other drugs (and their sources) included: cirazoline HCl (Synthélabo Recherche, France); clorgyline HCl (Sigma Chemical Co., U.S.A.); cocaine HCl (Alcaloides Abelló S.A., Spain); deprenyl (RBI, U.S.A.); desipramine HCl (Rorer Central Research, U.S.A.); diazepam (S.A. Lasa Laboratories, Spain); haloperidol HCl (Sigma); idazoxan HCl (synthesized by Dr F. Geijo at S.A. Lasa Laboratories); pargyline HCl (Sigma); reserpine (Serpasil) (Ciba-Geigy, Spain); Ro 41-1049 [N-(2-aminoethyl)-5-(3-fluorophenyl)-4-thiazole carboxamide] HCl, Ro 16-6491 [N-(2-aminoethyl-*p*-chlorobenzamide] HCl (F. Hoffmann-La Roche Ltd.). Other reagents were obtained from Sigma Chemical Co. (U.S.A.).

Results

In vivo *effects of MAO inhibitors and other psychotropic drugs on NAIBS*

Chronic treatment (14 days) with the irreversible MAO-A inhibitor clorgyline (0.3–10 mg kg^{-1}) markedly decreased the density of NAIBS in the rat cerebral cortex (Table 1 and Figure 1a). This effect appeared to be dose-dependent, at least in the range of 1, 3 and 10 mg kg^{-1}, and the total density of NAIBS decreased by 28%, 40% and 51% ($P<0.01$), respectively. In contrast, acute (1 day) treatment with clorgyline (1 mg kg^{-1}) did not alter the density of NAIBS in the brain (Table 1). Chronic treatment (14 days) with pargyline (10 mg kg^{-1}), an irreversible non-selective MAO inhibitor *in vivo* particularly after repeated treatment (see Fowler *et al.*, 1981), also markedly decreased (46%; $P<0.01$) the density of NAIBS in the cerebral cortex (Table 1 and Figure 1b). In contrast, chronic treatment (14 days) with Ro 41-1049 (1 mg kg^{-1}), a selective but reversible MAO-A inhibitor, did not decrease the density of NAIBS in the cerebral cortex (Table 1).

Acute (1 day) and/or chronic (14 days) treatments with the NA reuptake blockers desipramine (3 mg kg^{-1}) or cocaine (10 mg kg^{-1}) did not modify significantly the binding parameters of [^{3}H]-idazoxan to NAIBS in the cerebral cortex (Table 2). Moreover, chronic depletion of brain NA with reserpine (0.12 mg kg^{-1} for 14 days) resulted in similar negative results (Table 2). Other chronic treatments (14 days) with haloperidol (1 mg kg^{-1}) or diazepam (10 mg kg^{-1}) did not alter the density of NAIBS in the rat brain (Table 2).

In vitro *effects of MAO inhibitors and other psychotropic drugs on [^{3}H]-idazoxan and [^{3}H]-Ro 41-1049 binding*

Since clorgyline and pargyline irreversibly bind to the enzyme MAO, the possibility that the chronic *in vivo* effects of both drugs on NAIBS could be related, at least in part, to an interaction of [^{3}H]-idazoxan with the MAO was investigated. In the rat cerebral cortex, *in vitro* competition curves for clorgyline against [^{3}H]-idazoxan binding (in the presence of 10^{-6} M adrenaline) were clearly biphasic and nonlinear analyses revealed the existence of two distinct binding sites, one of them with very high affinity (40 picomolar) for clorgyline (Table 3 and Figure 2a). Interestingly, the MAO inhibitors pargyline and deprenyl, which like clorgyline possess a propargylamine structure (Youdim *et al.*, 1988), also displaced the binding of [^{3}H]-idazoxan from two distinct sites but with much lower potency (Table 3). In contrast, the structurally diverse compounds Ro 16-6491 (selective MAO-B inhibitor) and Ro 16-6491 (selective MAO-B inhibitor) and Ro 41-1049 (selective MAO-A inhibitor) displaced the binding of [^{3}H]-idazoxan to NAIBS monophasically and with very low potencies (Table 3 and Figure 2a).

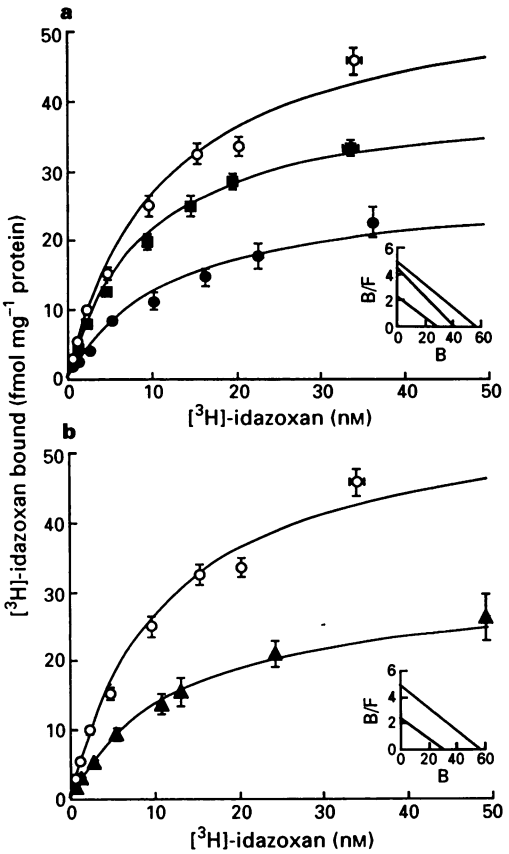

Figure 1 Specific binding of [^{3}H]-idazoxan (in the presence of 10^{-6} M adrenaline) to NAIBS in the rat cerebral cortex after saline (○) or chronic treatment with (a) clorgyline 1 mg kg^{-1} (■) or 10 mg kg^{-1} (●) and (b) pargyline 10 mg kg^{-1} (▲) i.p., every 12 h for 14 days. Each point of the saturation curve is the mean ± s.e.mean (vertical and horizontal bars) of 5 to 20 experiments. Inset: Scatchard plots (same data) showing K_d and B_{max} values similar to those obtained by nonlinear analysis. See Table 1 for changes in binding parameters and other details.

As expected, competition curves for idazoxan against [^{3}H]-idazoxan binding were monophasic and the best fit was to a single population of high affinity binding sites (Table 3 and Figure 2a). The various psychotropic drugs used in the chronic treatments (desipramine, reserpine, haloperidol and cocaine) also competed but with very low affinities against [^{3}H]-idazoxan binding to NAIBS (K_i values in the high micromolar range) (Table 3).

Competition curves for clorgyline and Ro 41-1049 against [^{3}H]-Ro 41-1049 binding to MAO-A were clearly monophasic and the best fit was to a single population of high affinity binding sites (K_i values: 0.18 nM and 22 nM, respectively) (Figure 2b). In contrast, idazoxan displayed very low affinity (K_i: 40 μM) against the binding of [^{3}H]-Ro 41-1049 to MAO-A (Figure 2b).

With these experiments a direct interaction of [^{3}H]-idazoxan with the active centre of MAO-A and B isoenzymes was discounted.

In vitro *preincubation with clorgyline and pargyline on NAIBS*

To further assess the mechanism of action involved in the *in vivo* down-regulation of NAIBS induced by clorgyline and pargyline, incubation experiments were performed to determine if, consistent with their actions on MAO, these compounds could also irreversibly bind to NAIBS. Thus, clorgyline and less potently pargyline, irreversibly blocked the binding of [^{3}H]-idazoxan to NAIBS. Preincubation of cortical membranes with 10^{-9} M or 10^{-6} M clorgyline reduced by 30% and 49% ($P<0.01$), respectively, the total density of NAIBS (Figure 3a). Similarly, preincubation with 10^{-6} M or 10^{-5} M pargyline also reduced by 17% and 32% ($P<0.01$), respectively, the density of NAIBS (data not shown). These preincubation procedures did not affect the affinity (K_d) of [^{3}H]-idazoxan binding to NAIBS (Figure 3a).

Next, the question was addressed whether in cortical membranes preincubated with 10^{-6} M clorgyline, the affinity of a potent and selective drug for NAIBS, cirazoline, as well as the proportion of high- and low-affinity sites of NAIBS were altered. Both in membranes preincubated in buffer only (control) and in those preincubated with 10^{-6} M clorgyline, competition curves for cirazoline against [^{3}H]-idazoxan binding (in the presence of 10^{-6} M adrenaline) were biphasic and, as expected (Olmos *et al.*, 1992), curve fitting with the LIGAND programme revealed that a two-site fit was significantly better than a one-site binding model (Figure 3b). Preincubation with 10^{-6} M clorgyline did not alter the affinity of cirazoline for the two populations of NAIBS, but significantly reduced (60%; $P<0.01$) the binding of [^{3}H]-idazoxan to the high affinity population (B_{maxH}) of NAIBS, without affecting the binding of the radioligand to the low affinity population (B_{maxL}) (Figure 3b).

In vivo *acute effects of clorgyline on NAIBS*

Chronic but not acute treatment with clorgyline (both with a 48 h period of drug washout) was found to induce down-regulation of NAIBS. However, since clorgyline and pargyline appeared to bind irreversibly *in vitro* to NAIBS, the effects of various acute treatments with high doses of clorgyline (10–80 mg kg^{-1}) were studied at shorter time intervals (6–48 h) to assess further whether this drug can induce a rapid and irreversible blockade of [^{3}H]-idazoxan binding to NAIBS *in vivo*. Clorgyline at doses of 10 mg kg^{-1} (the

Table 1 Effects of acute and/or chronic treatments with monoamine oxidase (MAO) inhibitors on NAIBS in the rat cerebral cortex

Treatment	Dose (mg kg^{-1})	[^{3}H]-idazoxan K_d (nM)	[^{3}H]-idazoxan B_{max} (fmol mg^{-1} protein)	n
Saline	–	11.3 ± 0.6	57 ± 2	20
Clorgyline				
Acute	1	15.8 ± 1.3	57 ± 2	4
Chronic	0.3	10.1 ± 1.2	34 ± 2*	6
	1	9.2 ± 1.0	41 ± 2*	8
	3	13.3 ± 1.9	34 ± 3*	6
	10	12.5 ± 1.8	28 ± 3*	6
Pargyline				
Chronic	10	12.9 ± 1.9	31 ± 5*	5
Ro 41-1049				
Chronic	1	11.6 ± 0.7	61 ± 4	5

Each drug was administered i.p., every 12 h for 14 days, except for the acute clorgyline treatment which was for only one day. The rats were killed 48 h after the last injection. Neural membranes were incubated at 25°C for 30 min with eight concentrations of [^{3}H]-idazoxan (6×10^{-10} M to 5×10^{-8} M). The specific binding of [^{3}H]-idazoxan to NAIBS was defined as the difference between binding in the presence of 10^{-6} M adrenaline (total nonadrenoceptor binding) and 10^{-4} M naphazoline (nonspecific binding). Binding parameters (K_d, B_{max}) were determined directly by computer-assisted nonlinear analysis from untransformed data using the EBDA-LIGAND programmes. Each value represents the mean ± s.e.mean of n experiments per group with an animal per experiment. One-way ANOVA followed by a multiple comparison test detected a significant decrease in B_{max} after chronic treatment with clorgyline (all doses) and pargyline, but not after acute clorgyline or chronic Ro 41-1049 treatments ($F[7,52] = 20.03$, $P = 0.0001$). *$P<0.01$ as compared with saline-treated group (ANOVA followed by Scheffés test).

Table 2 Effects of acute and/or chronic treatments with psychotrophic drugs on NAIBS in the rat cerebral cortex

Treatment	Dose (mg kg^{-1})	[^{3}H]-idazoxan K_d (nM)	[^{3}H]-idazoxan B_{max} (fmol mg^{-1} protein)	n
Saline	–	11.3 ± 0.6	57 ± 2	20
Desipramine				
Acute	3	13.4 ± 1.1	67 ± 5	4
Chronic	3	8.0 ± 0.3	61 ± 3	6
Cocaine				
Chronic	10	9.1 ± 0.3	54 ± 1	5
Reserpine				
Chronic	0.12	9.3 ± 0.7	57 ± 2	5
Haloperidol				
Chronic	1	11.5 ± 2.0	57 ± 5	3
Diazepam				
Chronic	10	9.8 ± 0.8	57 ± 2	5

Each drug, except reserpine, was administered i.p., every 12 h for 14 days or for only one day in the acute desipramine treatment. Reserpine was administered s.c., every 48 h for 14 days. The rats were killed 48 h after the last injection. Other details as for Table 1. Each value represents the mean ± s.e.mean of n experiments per group with an animal per experiment. One-way ANOVA did not detect any significant change in the binding parameters of [^{3}H]-idazoxan to NAIBS after the various treatments.

Table 3 Binding parameters of various drugs to NAIBS in the rat cerebral cortex

Drug	[^{3}H]-idazoxan K_{iH} (nM)	[^{3}H]-idazoxan K_{iL} (nM)	[^{3}H]-idazoxan $\%R_H$	n
MAO inhibitors				
Clorgyline	0.04	10,600	38	10
Pargyline	150	35,000	35	3
Deprenyl	1,400	55,000	76	3
Ro 16-6491	1,900	–	100	2
Ro 41-1049	79,000	–	100	2
Other drugs				
Idazoxan	14	–	100	3
Desipramine	6,400	–	100	2
Reserpine	12,100	–	100	2
Haloperidol	13,400	–	100	3
Cocaine	338,000	–	100	2

Cortical membranes were incubated at 25°C for 30 min with [^{3}H]-idazoxan (4×10^{-9} M in the presence of 10^{-6} M adrenaline) and in the absence or the presence of the competing drugs (10^{-14} M or 10^{-10} M to 10^{-3} M). Binding parameters (K_{iH}, K_{iL} and $\%R_H$, defined as the percent of high affinity sites for a given drug) were determined directly by simultaneous analysis of n independent experiments for each drug using the EBDA-LIGAND or the GraFit programmes. For the propargylamines (clorgyline, pargyline and deprenyl), computer-assisted curve fitting demonstrated that a two-site fit was significantly better than a one-site binding model ($P<0.001$, F test).

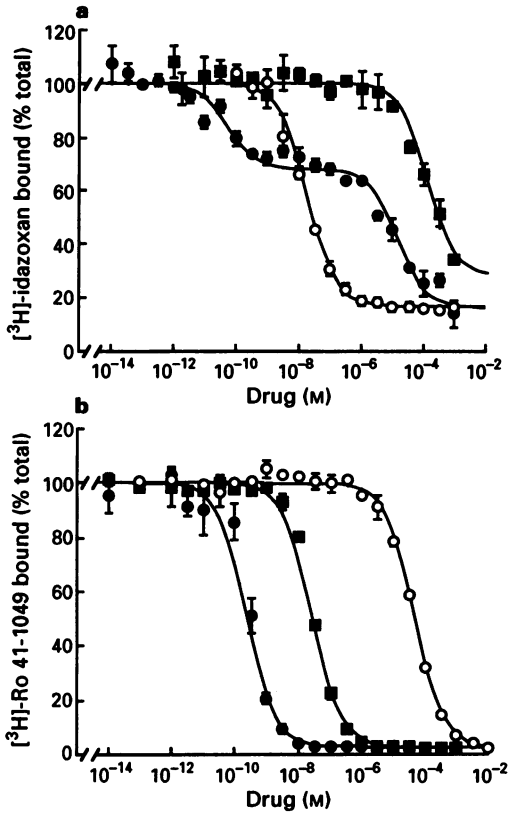

Figure 2 (a) Inhibition of binding of [^{3}H]-idazoxan (in the presence of 10^{-6} M adrenaline) to NAIBS in the rat cerebral cortex by clorgyline (●), idazoxan (○) and Ro 41-1049 (■). Cortical membranes were incubated at 25°C for 30 min with [^{3}H]-idazoxan (4×10^{-9} M) in the absence or presence of various concentrations of the competing drugs. Total control binding was 1,900 d.p.m. Data shown are mean ± s.e.mean (vertical bars) of 2 to 10 experiments per drug. See Table 3 for K_i values and other details. (b) Inhibition of binding of [^{3}H]-Ro 41-1049 to MAO-A in the rat cerebral cortex by clorgyline (●), Ro 41-1049 (■) and idazoxan (○). Cortical membranes were incubated at 37°C for 60 min with [^{3}H]-Ro 41-1049 (4.5×10^{-9} M) in the absence or presence of various concentrations of the competing drugs. Total control binding was 27,000 d.p.m. Data shown are mean ± s.d. of 2 experiments per drug. The K_i values were calculated as above by simultaneous analysis with the EBDA-LIGAND programmes: clorgyline, $K_i = 0.18$ nM; Ro 41-1049, $K_i = 22.4$ nM; and idazoxan, $K_i = 40{,}400$ nM.

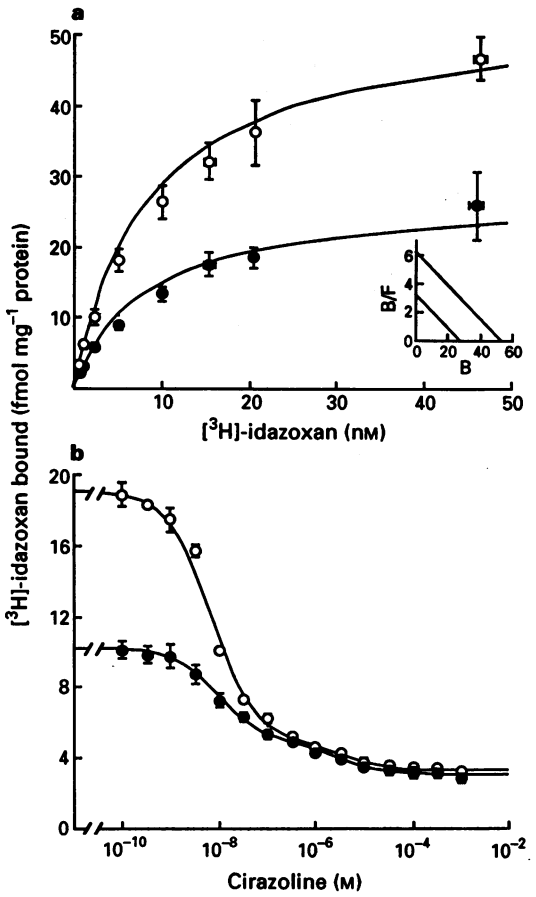

Figure 3 Specific binding of [^{3}H]-idazoxan (in the presence of 10^{-6} M adrenaline) to NAIBS in the rat cerebral cortex after preincubation (30 min at 25°C) of neural membranes in buffer only (○) or buffer containing 10^{-6} M clorgyline (●). (a) Saturation curves of [^{3}H]-idazoxan binding to NAIBS. Nonlinear analysis (EBDA-LIGAND programmes) of untransformed data yielded $K_d = 8.6 \pm 0.5$ nM; $B_{max} = 53 \pm 5$ fmol mg^{-1} of protein for control membranes ($n = 6$) and $K_d = 8.1 \pm 0.9$ nM; $B_{max} = 27 \pm 4$ fmol mg^{-1} of protein for membranes preincubated with clorgyline ($n = 6$) ($P < 0.01$ for B_{max} values). Each point is the mean ± s.e.mean (vertical bars) of n experiments per group. (b) Competition curves for cirazoline against [^{3}H]-idazoxan (4×10^{-9} M) binding to NAIBS. Computer-assisted curve fitting (EBDA-LIGAND programmes) demonstrated that in both sets of membranes a two-site fit was significantly better than a one-site binding model ($P < 0.0001$; F test). The binding parameters obtained were $K_{iH} = 5.2$ nM; $K_{iL} = 2{,}900$ nM; $B_{maxH} = 51$ fmol mg^{-1} of protein; $B_{maxL} = 6$ fmol mg^{-1} of protein for control membranes ($n = 3$) and $K_{iH} = 6.6$ nM; $K_{iL} = 1{,}300$ nM; $B_{maxH} = 20$ fmol mg^{-1} of protein; $B_{maxL} = 7$ fmol mg^{-1} of protein for clorgyline-treated membranes ($n = 6$). Simultaneous analysis of both sets of data with the GraFit programme yielded significant differences between the two groups only with respect to the B_{maxH} ($P < 0.01$; F test). Each point is the mean ± s.e.mean (vertical bars) of n experiments per group.

highest dose used in the chronic studies) and 20 mg kg^{-1} for 6, 12, 24 and 48 h did not induce any reduction of NAIBS in the cerebral cortex (Figure 4). In fact, only with very high doses of clorgyline (40 and 80 mg kg^{-1} for 6 and 48 h) was a modest decrease (21–28%) in the density of NAIBS in the cerebral cortex found (Figure 4).

Discussion

The binding of [^{3}H]-idazoxan to NAIBS has been shown to be markedly heterogeneous (Olmos *et al.*, 1992) and the existence of two affinity states for these sites has been proposed (Wikberg *et al.*, 1992). In this context, the results of the competition experiments for clorgyline (as well as for pargyline and deprenyl) against [^{3}H]-idazoxan binding to NAIBS indicate a biphasic interaction between these propargylamines and these sites, with an apparent very high affinity (40 picomolar) displayed by clorgyline for a relevant population of NAIBS in the brain. These results could be explained, as the incubation experiments with these MAO-inhibitors suggested, by an irreversible interaction of clorgyline and pargyline with only one population of NAIBS, and then by a competitive low affinity binding to the remaining population of non-adrenoceptor sites. Thus, clorgyline has been shown to bind irreversibly to a high affinity population of NAIBS (B_{maxH}) that appears to represent a 90% of total non-adrenoceptor [^{3}H]-idazoxan binding (see Figure 3b). In saturation experiments the reduction of [^{3}H]-idazoxan binding after preincubation with 10^{-6} M clorgyline (total density of NAIBS reduced by about 40%, see Figure 3a) was similar to the decrease (53%) of [^{3}H]-idazoxan binding to only the high affinity population of NAIBS found in competition experiments with cirazoline (Figure 3b), which in addition was also similar to the 40% inhibition induced by clorgyline (10^{-6} M) in competition experiments (Figure 2a). Therefore, clorgyline *in vitro* appears to interact irreversibly with the high affinity population of NAIBS in the brain.

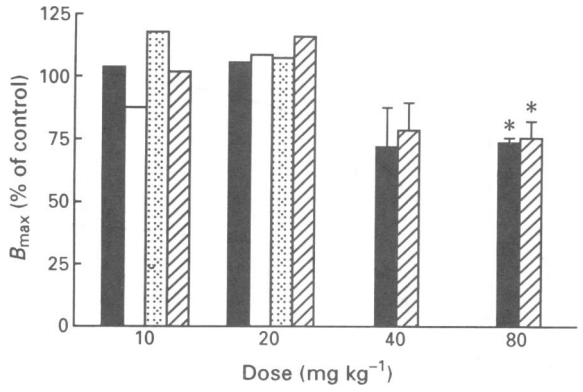

Figure 4 Effect of acute clorgyline administration on the density of NAIBS in the rat cerebral cortex expressed as a percentage of that in saline-treated rats. Total density of NAIBS (B_{max}) was determined through the specific binding of [^{3}H]-idazoxan (in the presence of 10^{-6} M adrenaline) at 6 h (solid column), 12 h (open column), 24 h (stippled column) and 48 h (hatched column) after a single administration (i.p.) of clorgyline at the doses indicated. Values for the doses of 10 and 20 mg kg^{-1} are from one single experiment and for the doses of 40 and 80 mg kg^{-1} represent the mean ± s.d. of two experiments with an animal per experiment. One-way ANOVA followed by a multiple comparison test detected a significant decrease in B_{max} at 6 and 48 h after the administration of 80 mg kg^{-1} of clorgyline ($F[2,6] = 9.19$; $P = 0.015$). *$P < 0.05$ as compared to saline-treated group (ANOVA followed by Scheffé's test).

Since the molecular structure of clorgyline does not resemble that of imidazoli(di)nes, it raises the question as to whether the high affinity found for this MAO-inhibitor on NAIBS is the result of further interactions of [^{3}H]-idazoxan with other protein/binding sites. In this respect, NAIBS appear to be associated with mitochondrial outer membranes and the density of these sites correlates well with MAO activity (Tesson *et al.*, 1991). However, the low affinity on NAIBS displayed by the selective (not propargylamines) MAO-A and MAO-B inhibitors, Ro 41-1049 (Cesura *et al.*, 1990a; Da Prada *et al.*, 1990) and Ro 16-6491 (Cesura *et al.*, 1988; Cesura *et al.*, 1990b), respectively; the lack of down-regulation of NAIBS induced by chronic treatment with Ro 41-1049; and the much higher densities (40 to 75 times) of the two MAO isoenzymes (Saura *et al.*, 1992) with respect to that of NAIBS in the rat cerebral cortex, makes it unlikely that the portion of binding of [^{3}H]-idazoxan blocked with very high affinity by clorgyline could be to enzyme MAO.

Recently, it has been reported that clorgyline also displays high affinity for the σ (sigma) binding sites in the mouse brain (Itzkak & Kassim, 1990). However, the possibility of a direct link/interaction between [^{3}H]-idazoxan and the σ site was also discarded. Firstly, the subcellular localization of the σ binding sites is different from that of NAIBS (Samovilova & Vinogradov, 1992). Secondly, haloperidol, which is a non selective, high affinity σ_1 and σ_2 ligand (Ferris *et al.*, 1991; Quirion *et al.*, 1992; Barnes *et al.*, 1992) displayed very low affinity for NAIBS (K_i = 13,400 nM); and thirdly, chronic treatment with haloperidol decreases the density of σ binding sites in the mouse and rat brains (Itzhak & Alerhand, 1989; Jansen *et al.*, 1992) but it does not affect the density of NAIBS in the rat brain (see Tables 2 and 3).

In addition, the autoradiographic distribution of NAIBS in the rat brain was recently found to be similar to that of peripheral type benzodiazepine sites (Mallard *et al.*, 1992). This site is also a mitochondrial protein which can be labelled by [^{3}H]-diazepam and other selective ligands (for a review see Gavish *et al.*, 1992). Also in this case a hypothetical link between NAIBS and this site was discarded. Thus, the compound PK 11195, a highly selective drug for the peripheral type benzodiazepine sites, displayed very low affinity (K_i > 10,000 nM) competing with the binding of [^{3}H]-idazoxan in human and rabbit liver mitochondria (Tesson *et al.*, 1991). Moreover, chronic treatment with diazepam did not alter the density of NAIBS in the rat brain (see Table 2).

Together these results suggest that, *in vitro*, clorgyline and to a lesser extent pargyline, directly bind to a high affinity population of NAIBS or alternatively that the binding of these MAO-inhibitors to a different protein (MAO, σ site or others) provokes an interaction of this protein with NAIBS that blocks the binding of [^{3}H]-idazoxan to these non-adrenoceptor sites.

The present study also demonstrates that chronic treatment (14 days) with clorgyline (0.3–10 mg kg^{-1}) and pargyline (10 mg kg^{-1}) markedly down-regulate the density of NAIBS in the rat brain. In contrast, various acute treatments with clorgyline (1–20 mg kg^{-1}) for different time intervals (6–48 h) did not alter the density of NAIBS. In the acute treatments only very high doses of clorgyline (40 and 80 mg kg^{-1}) produced significant but modest decreases in the density of NAIBS. These results suggest that the irreversible binding of clorgyline and pargyline to NAIBS found *in vitro* is not entirely responsible for the decrease found *in vivo* after chronic treatment with low/moderate doses of both drugs, and it raises the question as to whether these drugs can act as 'direct agents' upon these non-adrenoceptor sites and thus are able to decrease their density after sustained activation, or if the observed down-regulation of NAIBS is the result of an indirect mechanism. Although chronic treatment with the MAO inhibitor clorgyline has been shown to down-regulate brain α_2-adrenoceptors probably by increasing synaptic NA availability (Giralt & García-Sevilla, 1989 and other references therein), the integrity and/or parallel modulation of the α_2-adrenoceptor is not necessary for the regulation of NAIBS (Olmos *et al.*, 1992). Moreover, the effect of clorgyline on the density of NAIBS in the brain does not appear to be dependent on NA availability since chronic treatment with other drugs (Ro 41-1049, desipramine, cocaine and reserpine) which also potently modify the intraneuronal and/or synaptic NA pools did not induce any significant effect on NAIBS. Another possibility is that some non identified metabolite(s) of the propargylamines clorgyline and pargyline could be active on NAIBS, but very little is known about the metabolism of these compounds (Fowler *et al.*, 1981).

Together the results are compatible with the view that clorgyline and pargyline, through an as yet unknown *in vivo* mechanism, can modulate the density of NAIBS in the brain. Although the native ligand and functions of NAIBS are also unknown, the observed down-regulation of NAIBS induced specially by clorgyline could be related to the antidepressant effects of this drug. In this context, the findings of increased NAIBS in the brain of depressed suicide victims (Barturen *et al.*, 1992) as well as elevated non-adrenoceptor binding sites labelled by [^{3}H]-*p*-aminoclonidine in platelet from depressed patients that can be down-regulated by desipramine (Piletz *et al.*, 1991), provide further evidence that these putative imidazoline receptors may be involved in the pathophysiology of certain forms of depression. In contrast, NAIBS do not appear to be involved with the chronic effects of other psychotropic drugs such as haloperidol, diazepam and cocaine.

The authors wish to thank Dr J.G. Richards and Dr J. Saura (F. Hoffmann-La Roche Ltd., Switzerland) as well as the various pharmaceutical firms for gifts of drugs. This work was supported by DGICYT Grant PM 91-0069 and by a Grant from S.A. LASA Laboratorios, Barcelona, Spain. Part of this work was presented at the 1st International Symposium on Imidazoline Preferring Receptors, Paris, France, June 1992.

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(Received August 3, 1992
Revised October 12, 1992
Accepted October 14, 1992)