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ORIGINAL ARTICLE

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Effects of imidazoline receptor ligands on monoamine synthesis in the rat brain in vivo

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Abstract This study was designed to assess the effects of imidazoline drugs on putative presynaptic imidazoline receptors modulating brain monoamine synthesis in vivo. The accumulation of 3,4-dihydroxyphenylalanine (dopa) and 5-hydroxytryptophan (5-HTP) after decarboxylase inhibition was used as a measure of the rate of tyrosine and tryptophan hydroxylation in various brain regions of naive rats and after irreversible α_2-adrenoceptor inactivation with EEDQ (1.6 mg/kg, i.p., 6 h).

Clonidine (1–3 mg/kg), moxonidine (1–10 mg/kg) and rilmenidine (10 mg/kg) (mixed I_1/α_2 agonists) decreased dopa and 5-HTP synthesis in the cerebral cortex (14%–81%), hippocampus (27%–84%) and/or striatum (29%–56%), but these inhibitory effects were abolished in *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)-treated rats. Similarly, the stimulatory effect of efaroxan (mixed I_1/α_2 antagonist; 10 mg/kg) on dopa synthesis in the cortex (77%) and hippocampus (57%) was abolished by EEDQ. The selective I_1-ligand 2-endo-amino-3-exo-isopropylbicyclo-heptane (AGN-192403; 5–10 mg/kg) did not modify dopa or 5-HTP synthesis in any brain region in naive or EEDQ-treated rats. Idazoxan (mixed I_2/α_2 antagonist; 20 mg/kg) increased dopa synthesis in the cortex (111%) and hippocampus (87%), but the stimulatory effects were abolished by EEDQ. Moreover, idazoxan and efaroxan decreased 5-HTP synthesis in the cortex (12%–34%) and hippocampus (30%–34%) in a manner sensitive to blockade by the 5-HT_{1A} receptor antagonist WAY 100135. The selective I_2-ligands 2-(2-benzofuranyl)-2-imidazoline (2-BFI; 20 mg/kg) and 2-styryl-2-imidazoline (LSL 61122; 10 mg/kg) did not alter the synthesis of dopa or 5-HTP in the cortex or hippocampus. In striatum, 2-BFI (1–20 mg/kg) dose-dependently decreased dopa synthesis (ED_{50}: 5.9 mg/kg), reduced dopamine levels (6%–36%) and increased those of its metabolites DOPAC (15%–95%) and HVA (24%–74%). The inhibitory effect of 2-BFI on dopa/dopamine synthesis in striatum remained unchanged after alkylation of imidazoline receptors with isothiocyanatobenzyl imidazoline (IBI; 60 mg/kg, 6 h) or blockade of these receptors with 2-(2-ethyl 2,3-dihydro-2-benzofuranyl)-2-imidazole (KU-14R; 7–20 mg/kg).

Therefore, most imidazoline drugs modulated the synthesis of brain monoamines through interaction with α_2-adrenoceptors or 5-HT_{1A} receptors. The results do not provide functional evidence for the existence of presynaptic imidazoline receptors regulating the synthesis of monoamines in the rat brain.

Key words Imidazoline receptors · α_2-adrenoceptors · Dopa/noradrenaline synthesis · Dopa/dopamine synthesis · 5-HTP/serotonin synthesis · Rat brain

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Introduction

In the central nervous system (CNS) the functions associated with imidazoline receptors are largely unknown but evidence exists for their involvement in various CNS disorders (for a review see García-Sevilla et al. 1999). The density of I_1-imidazoline binding sites and imidazoline receptor proteins are elevated in major depression, whereas I_2-imidazoline receptors are increased in Alzheimer's disease and decreased in Huntington's disease (Piletz et al. 1991, 1996; García-Sevilla et al. 1996, 1998; Reynolds et al. 1996). Moreover, treatments with cyclic antidepressant drugs and MAO inhibitor antidepressants resulted in down-regulation of imidazoline receptor binding sites or proteins (Alemany et al. 1995; Piletz et al. 1996; Zhu et al. 1997), suggesting that imidazoline receptors interact functionally with brain monoamine systems.

Recently, the presence of imidazoline receptors in axon terminals of the rat CNS (Ruggiero et al. 1998) suggests a possible role for these receptors in neurotransmitter release and synthesis. Presynaptic imidazoline receptors

modulate the release of noradrenaline from peripheral, but not central, noradrenergic neurones (Göthert et al. 1995; Molderings et al. 1997). However, a stimulatory effect of imidazoline receptor ligands on noradrenaline release was observed in the cerebral cortex which was mediated by an indirect extra-cortical mechanism (Meana et al. 1997).

In adrenal chromaffin cells, imidazoline receptor ligands have been shown to stimulate the expression (mRNA) of the enzyme tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of catecholamines (Evinger et al. 1995). In the brain, the synthesis of monoamines is modulated by presynaptic α_2-adenoceptors, which operate through the negative control of the rate-limiting enzymes tyrosine and tryptophan hydroxylases (Andén et al. 1976; Esteban et al. 1996). The pharmacological properties of the α_2-autoreceptors and α_2-heteroreceptors modulating monoamine synthesis have been studied using clonidine and idazoxan as the prototypical agonist and antagonist at α_2-adrenoceptors (Pi and García-Sevilla 1992; Esteban et al. 1996). However, these drugs and other compounds (e.g. oxymetazoline, guanoxabenz, efaroxan) used in the characterization of the monoamine synthesis-modulating α_2-adrenoceptors also recognize with high affinity the various types of imidazoline receptors (Ernsberger et al. 1987; Michel et al. 1989; Bricca et al. 1993; De Vos et al. 1994).

Therefore, the aim of the present study was to assess the role of imidazoline receptors modulating monoamine synthesis in the rat brain in vivo. In order to unmask possible effects of adrenergic drugs on imidazoline receptors, the alkylating agent EEDQ was used as a tool to inactivate α_2-adrenoceptors (Miralles et al. 1993b; Pineda et al. 1993; Meana et al. 1997). Preliminary accounts of this work were given at the Third International Symposium on Imidazoline Receptors, Bonn, Germany, and at the 29th annual meeting of the Spanish Society of Physiological Sciences, Liverpool, UK (Esteban et al. 1998; Sastre-Coll et al. 1998).

Materials and methods

Animals and treatments. Male Sprague-Dawley rats (240–280 g) were used. The animals were housed in groups of four under controlled environmental conditions (temperature 22 ± 2 °C; humidity 60%–70%; 12-h light/dark cycle) with free access to standard food and tap water.

Groups of rats were treated intraperitoneally (i.p.) with the following drugs and dosage regimens: clonidine (1 and 3 mg/kg), AGN-192403 (5 and 10 mg/kg), moxonidine (1 and 10 mg/kg), rilmenidine (10 mg/kg), efaroxan (10 mg/kg), idazoxan (20 mg/kg), 2-BFI (1, 3, 7, 10 and 20 mg/kg), LSL 61122 (10 mg/kg). In these acute treatments a single dose of the drug was administered and the rats were sacrificed 1 h after the injection (i.e. the drug was injected 30 min before NSD 1015, see below, and the animal was decapitated after another 30 min). In some experiments, rats were pretreated with a single dose of WAY100135 (10 mg/kg, i.p.), a

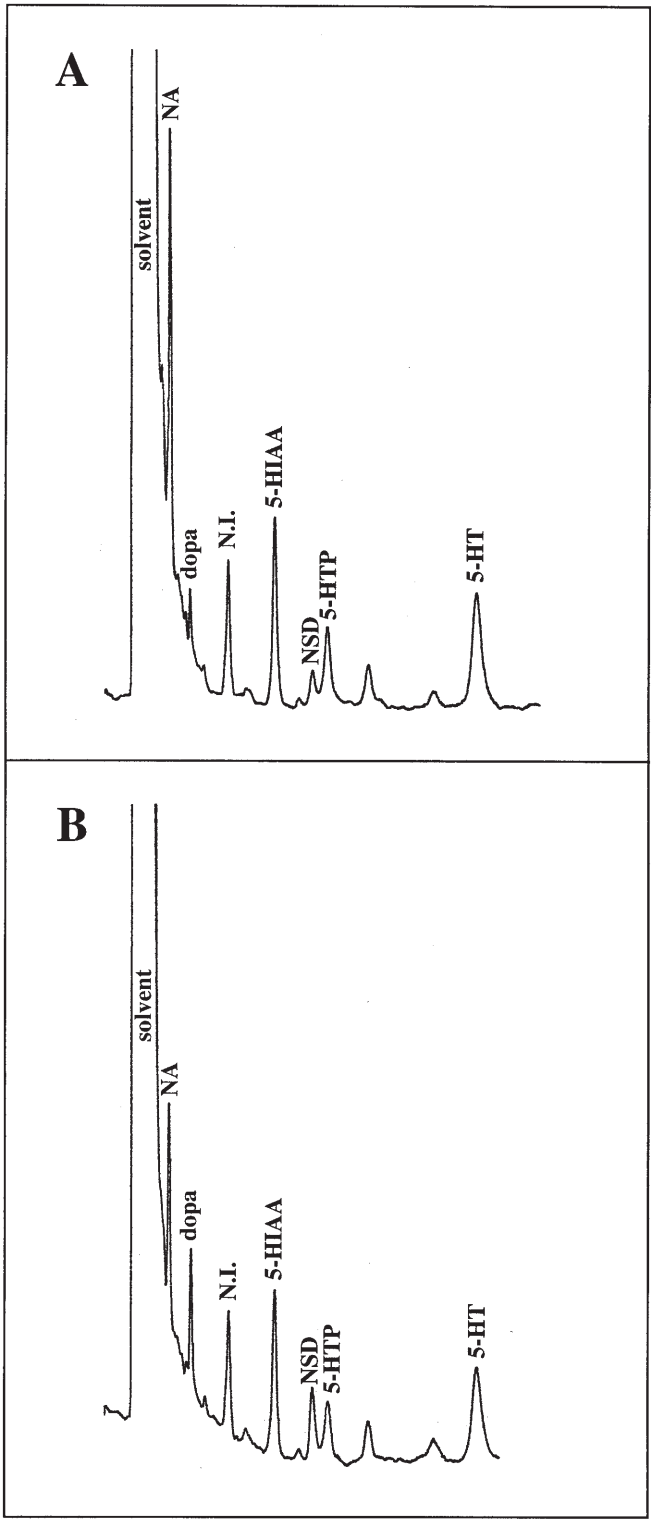

Fig. 1 Representative chromatographic (HPLC-ED) analyses of precursor amino acids, monoamines and metabolites in hippocampal tissue from a vehicle-treated rat (**A**) and an EEDQ (1.6 mg/kg, i.p., 6 h)-treated rat (**B**). The retention times for the various compounds (identical to those in the standard mixture) were (in min): 2.8 (*NA* noradrenaline), 3.7 (*dopa*), 7.2 (*5-HIAA* 5-hydroxyindoleacetic acid), 9.4 (*5-HTP*) and 15.8 (*5-HAT*). *N.I.* (compound not identified), *NSD* (NSD 1015). **A** Values in ng/g tissue: 326 (*NA*), 85 (*dopa*), 259 (*5-HIAA*), 160 (*5-HTP*) and 279 (*5-HT*). **B** Values in ng/g tissue: 176 (*NA*), 192 (*dopa*), 261 (*5-HIAA*), 154 (*5-HTP*) and 250 (*5-HT*). Note that EEDQ increased the level of dopa and decreased that of NA. EEDQ did not alter significantly the levels of 5-HTP or 5-HT. See text for further details

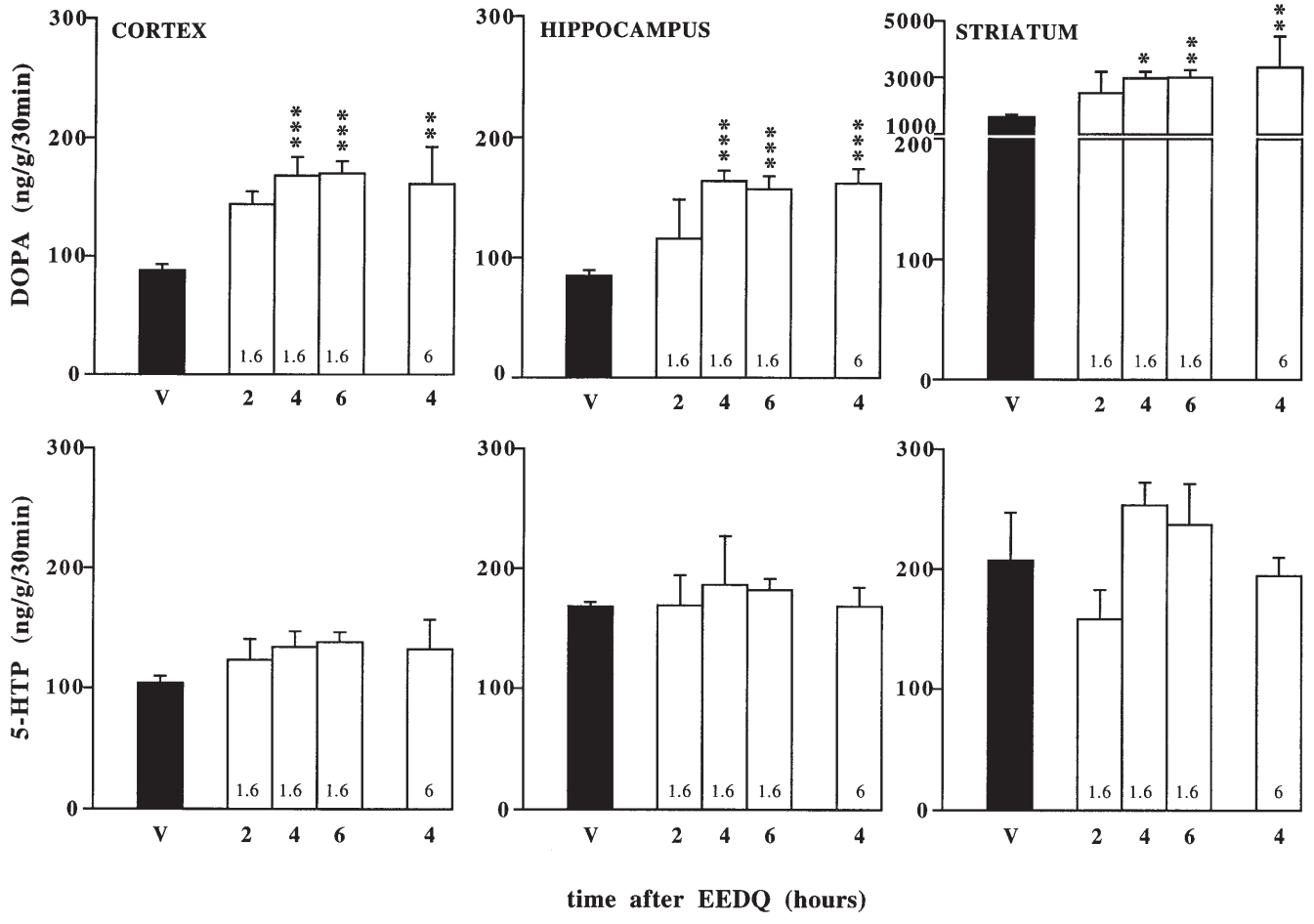

Fig. 2 Effects of two doses of EEDQ (1.6 mg/kg and 6 mg/kg) at different time intervals (2, 4 and 6 h) on dopa and 5-HTP accumulation in the rat cerebral cortex, hippocampus and striatum. Vehicle control rats received the same volume of vehicle (i.p.); 30 min before sacrifice rats received the NSD 1015. *Bars* represent means ± SEM derived from 3–8 experiments for each drug. One-way ANOVA followed by Scheffé or Fisher LSD (least statistical deviation) were used for statistical evaluation. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 when compared with the vehicle control group

selective 5-HT_{1A} receptor antagonist, 5 min before the administration of efaroxan (10 mg/kg, i.p.) or 2-BFI (7 mg/kg, i.p.). In other experiments, rats were pretreated with a single dose of dizocilpine (0.1 mg/kg, i.p.), a specific NMDA receptor antagonist, 5 min before the administration of 2-BFI (7 mg/kg, i.p.). The doses of the various drugs were chosen from previous studies demonstrating clear-cut effects on the synthesis of monoamines (Pi and García-Sevilla 1992; Esteban et al. 1996; Lladó et al. 1996).

To assess the effects of these drugs on tyrosine and tryptophan hydroxylase activity after α_2-adrenoceptor inactivation, various groups of animals were pretreated with the alkylating agent EEDQ. Low doses of EEDQ (0.25–1.6 mg/kg) have been shown to induce an almost complete reduction (> 90% at 2–4 h) in the density of brain α_2-adrenoceptors (Miralles et al. 1993b). The treatment with higher doses of EEDQ (6–10 mg/kg) was shown to induce also a substantial reduction (60%–75% at 4–24 h) in the density of brain 5-HT_{1A} and dopamine D_2 receptors (Pinto and Battaglia 1994; Meller et al. 1988). The optimal dose and time for the pretreatment with EEDQ were first determined. Rats were treated with EEDQ (1.6 mg/kg and 6 mg/kg, i.p.; dissolved in ethanol and then diluted with propylene glycol/water in a final volume ratio of 1:1:2) or injected with vehicle, and sacrificed 2, 4 or 6 h after the injection (i.e. EEDQ was injected 90, 210 or 330 min before NSD 1015 and the animal was decapitated after another 30 min). From these experiments (see Figs. 1, 2) the dose of 1.6 mg/kg for 6 h was chosen. Then the animals were injected with the various drugs (see above) 5 h after EEDQ administration (1.6 mg/kg) and finally with NSD 1015 as above.

In order to further investigate the acute effects of 2-BFI in the striatum, groups of animals were pretreated with the alkylating agent for imidazoline receptors isothiocyanatobenzyl imidazoline (IBI; 60 mg/kg, i.p.) (Boronat et al. 1998) 5 h before 2-BFI (7 mg/kg). In other series of experiments, animals were pretreated with the imidazoline receptor antagonist 2-(2-ethyl 2,3-dihydro-2-benzofuranyl)-2-imidazole (KU-14R; 7 mg/kg and 20 mg/kg, i.p.) (Chan et al. 1997) 30 min before 2-BFI (7 mg/kg). All the animals received NSD 1015 (100 mg/kg, i.p.) 30 min before the sacrifice. This study was approved by the Dirección General de Investigación Científica y Técnica, Spain, research and ethical review board, and the experiments in rats were performed according to the guidelines of the Universitat de les Illes Balears.

Tyrosine hydroxylase activity (synthesis of dopa) and tryptophan hydroxylase activity (synthesis of 5-HTP). The in-vivo activity of tyrosine hydroxylase (tyrosine-3-monoxygenase; EC 1.14.16.2) and tryptophan hydroxylase (tryptophan-5-monoxygenase; EC 1.14.16.4), rate-limiting enzymes in the pathway for the synthesis of catecholamines and 5-HT, respectively, was determined simultaneously by measuring the accumulation of 3,4-dihydroxyphenylalanine (dopa) and 5-hydroxytryptophan (5-HTP) within 30 min after inhibition of the aromatic L-amino acid decarboxylase by a maximally effective dose of NSD 1015 (3-hydroxybenzylhydrazine HCl; 100 mg/kg, i.p.) (Carlsson et al. 1972; see also Nissbrandt et al. 1988). The dopa and 5-HTP accumulation method is the most commonly used assay system to monitor the in-vivo rate

of tyrosine and tryptophan hydroxylation in the brain. The dopa and 5-HTP formed from endogenous tyrosine and tryptophan, respectively, were then determined by high-pressure liquid chromatography (HPLC) with electrochemical detection (ED). The synthesis of dopa and 5-HTP was measured in brain regions enriched in noradrenaline and 5-HT (cerebral cortex and hippocampus) and dopamine (striatum) nerve terminals. The striatum is enriched also in 5-HT nerve terminals. However, only dopa accumulation was quantitated in this region after the different drug treatments because the optimal processing for dopa requires small aliquots (about 5 µl) of brain samples which were insufficient to measure 5-HTP/5-HT content. 5-HTP synthesis in the striatum was only measured after EEDQ treatments (see Fig. 2).

Brain samples. The rats were killed by decapitation and the brains were quickly removed and dissected on an ice-cold plate into the parieto-occipital cortex, hippocampus and striatum. Fresh brain regions were weighed and placed individually into cold tubes which contained 1 ml of 0.4 mol/l $HClO_4$, 0.01% K_2EDTA and 0.1% $Na_2S_2O_5$ and were homogenized with an Ultra-Turrax homogenizer (Type Tp 18/10). The samples were centrifuged at 40,000 *g* for 15 min at 4 °C and then the supernatant was filtered through 0.45-mm teflon syringe filters (Spartan-3; Aldrich Chemical, Milwaukee, Wis., USA) and various aliquots were injected into the HPLC system for the simultaneous determination of dopa, dopamine, noradrenaline, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-HTP and 5-HT.

Chromatographic analyses. The levels of precursor amino acids, monoamines and metabolites in the parieto-occipital cortex, hippocampus and striatum were determined by HPLC-ED as described previously (Adell et al. 1991; Esteban et al. 1996) (see Fig. 1 for representative chromatograms in the hippocampus). Aliquots (5–10 µl) of the purified supernatants from brain homogenates were subjected to HPLC on a Spherisorb S3 ODS1 C18 reversed-phase column (3-µm particle size range, 4.6 mm × 10 cm) coupled to a Tracer ODS2 C18 (2–5 µm particle size range) pre-column (Teknokroma). The mobile phase consisted of 0.1 mol/l KH_2PO_4; 2.1 mmol/l octane sulphonic acid; 0.1 mmol/l K_2EDTA and 12% (v/v) methanol (pH 2.7–2.8, adjusted with 85% H_3PO_4) and was pumped at a flow rate of 0.8 ml/min with a Waters M-510 solvent delivery system. The compounds were detected electrochemically by means of a cell with a glassy carbon working electrode with an applied potential of +0.75 V against an Ag/AgCl reference electrode (Waters 460 Electrochemical Detector). The current produced was monitored using a Waters M-743 data module. The concentrations of dopa, dopamine, noradrenaline, DOPAC, HVA, 5-HTP and 5-HT in a given sample were calculated by interpolating the corresponding peak height into a parallel standard curve.

Drugs. The following drugs were used: 2-endo-amino-3-exo-isopropylbicyclo (2.2.2) heptane HCl (AGN-192403; Research Biochemicals International, RBI, Natick, Mass., USA); 2-(2-benzofuranyl)-2-imidazoline HCl (2-BFI; synthesized and coded as LSL 61103 by Dr. F. Pla at Lasa Laboratorios, Barcelona, Spain); clonidine HCl (Boehringer, Ingelheim, Germany); dizocilpine maleate (formerly termed MK-801; RBI, Natick, Mass., USA); *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), efaroxan HCl (Sigma Chemical, St. Louis, Mo., USA); isothiocyanatobenzyl imidazoline HCl (IBI; a gift of Dr. D.D. Miller, University of Tennessee, Memphis, Tenn., USA); idazoxan HCl (synthesized by Dr. F. Geijo at Lasa Laboratorios); 2-(2-ethyl 2,3-dihydro-2-benzofuranyl)-2-imidazole HCl (KU-14R; synthesized and coded as LSL 90113 by Dr. F. Pla at Lasa Laboratorios); 2-styryl-2-imidazoline HCl (synthesized and coded as LSL 61122 by Dr. F. Geijo at Lasa Laboratorios); moxonidine HCl (Beiersdorf, Hamburg, Germany); 3-hydroxybenzyl-hydrazine HCl (NSD 1015; Sigma Chemical); phenelzine sulphate (Sigma Chemical); rilmenidine dihydrogenphosphate (Servier, Courbevoie, France) and *N*-tert-butyl-3-(4-(2-methoxyphenyl)-1-piperazinyl)-2-phenylpropanamide HCl (WAY100135; Wyeth Research, Taplow, UK). Other reagents were obtained from Sigma Chemical (USA).

Statistics. Results are expressed as means ± SEM. One-way ANOVA followed by Scheffé's or Fisher's LSD (least statistical deviation) test and Student's *t*-test were used for statistical evaluation. The level of significance was set at $P < 0.05$.

Results

Effects of irreversible inactivation of α_2-adrenoceptors by EEDQ on brain tyrosine and tryptophan hydroxylase activity in vivo

The irreversible inactivation of α_2-adrenoceptors by EEDQ (1.6 mg/kg, i.p.) caused a pronounced time-dependent increase in the synthesis of dopa in the cerebral cortex (64%, 92% and 94%), hippocampus (36%, 93% and 84%) and striatum (53%, 86% and 87%) at 2, 4 and 6 h after the alkylating agent, respectively (Figs. 1, 2). A higher dose of EEDQ (6 mg/kg, i.p., for 4 h) also elicited a significant increase in dopa synthesis in the cerebral cortex (83%), hippocampus (90%) and striatum (109%) (Fig. 2). In contrast, EEDQ (1.6 mg/kg and 6 mg/kg) did not alter significantly the synthesis of 5-HTP in any brain region (Figs. 1, 2). These results confirmed and extended those of a previous study (Esteban et al. 1996) which revealed the existence of a powerful inhibitory tone of α_2-autoreceptors on tyrosine hydroxylase activity, but not of α_2-heteroreceptors on tryptophan hydroxylase activity.

EEDQ treatment (1.6 mg/kg or 6 mg/kg for 2, 4 or 6 h) also reduced the levels of noradrenaline in the cerebral cortex (41%–67%, $P < 0.001$) and hippocampus (37%–68%, $P < 0.001$) (Fig. 1) and those of dopamine in the striatum (18%–30%, $P < 0.01$). However, EEDQ (1.6–6 mg/kg for 2–6 h) did not alter significantly the levels of 5-HT in any brain region (data not shown) (see Fig. 1).

From these experiments, a specific dose and time regimen for EEDQ (1.6 mg/kg for 6 h) was chosen to assess the effects of various imidazoline receptor ligands on tyrosine and tryptophan hydroxylase activity after irreversible inactivation of brain α_2-adrenoceptors.

Effects of I_1-imidazoline ligands on brain tyrosine and tryptophan hydroxylase activity in vivo

In naive rats, the acute administration of clonidine (mixed I_1/α_2 agonist; 1 mg/kg and 3 mg/kg, i.p.) decreased the synthesis of dopa in the cerebral cortex (34% and 79%), hippocampus (55% and 84%) and striatum (47% and 56%) (Fig. 3). However, the decreases induced by clonidine on dopa synthesis in naive rats were completely abolished in rats pretreated with EEDQ (Fig. 3). Similarly, moxonidine (mixed I_1/α_2 agonist; 1 mg/kg and 10 mg/kg, i.p.) decreased the synthesis of dopa in the cerebral cortex (14% and 36%), hippocampus (27% and 48%) and striatum (29% and 43%), but it failed to do so in EEDQ-pretreated rats (Fig. 3). Rilmenidine (another mixed I_1/α_2 agonist; 10 mg/kg, i.p.) did not modify significantly the synthesis of dopa in the cerebral cortex and hippocampus, but the drug decreased dopa accumulation in striatum (24%)

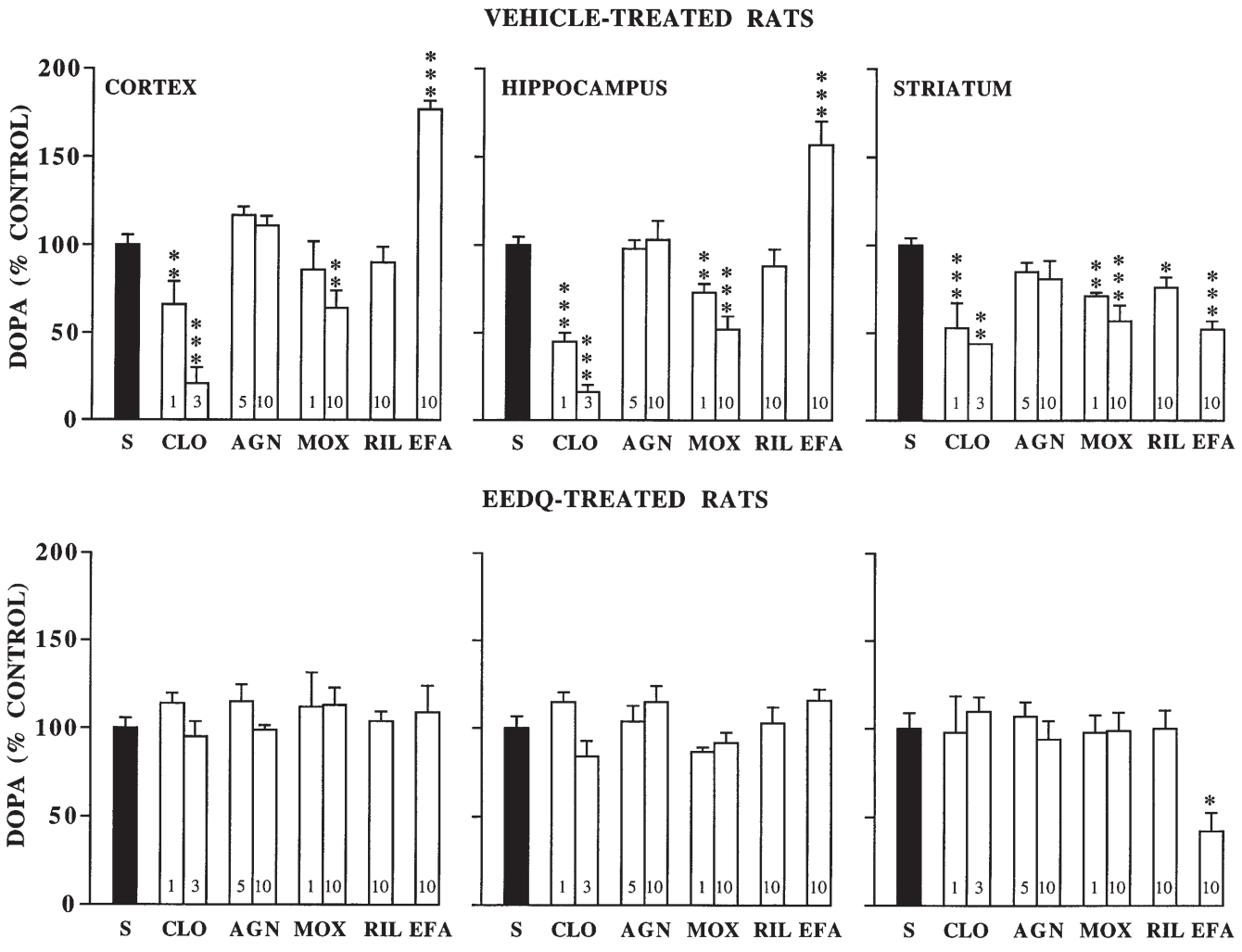

Fig. 3 Acute effects (1 h) of several I_1-imidazoline drugs on dopa accumulation in the cerebral cortex, hippocampus and striatum of naive rats and rats pretreated for 5 h with EEDQ (1.6 mg/kg). Clonidine (*CLO*; 1–3 mg/kg), AGN-192403 (*AGN*; 5–10 mg/kg), moxonidine (*MOX*; 1–10 mg/kg), rilmenidine (*RIL*; 10 mg/kg), efaroxan (*EFA*; 10 mg/kg). Saline controls (*S*) received the same volume of saline (i.p.) 30 min before NSD 1015 and then were sacrificed after another 30 min. [Mean control values in ng/g 30 min in naive rats: dopa 89 ± 5, n = 13 (cortex), 86 ± 4, n = 14 (hippocampus) and 1562 ± 67, n = 15 (striatum); in rats pretreated with EEDQ: dopa 170 ± 10, n = 8 (cortex), 157 ± 11, n = 6 (hippocampus) and 3005 ± 268, n = 8 (striatum)]. *Bars* represent means ± SEM derived from 3–8 experiments for each drug as percentage of saline-treated controls. One-way ANOVA followed by Scheffé or Fisher LSD (least statistical deviation) were used for statistical evaluation. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ when compared with the saline control group

which was abolished in EEDQ-pretreated rats (Fig. 3). The selective I_1-imidazoline ligand AGN-192403 (5 mg/kg and 10 mg/kg, i.p.) did not modify dopa synthesis in any brain region in naive or EEDQ-pretreated rats (Fig. 3).

The acute administration of efaroxan (mixed I_1/α_2 antagonist; 10 mg/kg, i.p.) increased dopa accumulation in both cerebral cortex (77%) and hippocampus (57%) (Fig. 3). In marked contrast, efaroxan decreased dopa formation in striatum (48%). The increase induced by efaroxan on dopa synthesis in the cerebral cortex and hippocampus of naive rats was abolished in rats pretreated with EEDQ. However, the decrease induced by efaroxan on dopa synthesis in striatum was unaffected by EEDQ pretreatment (58%; Fig. 3). Efaroxan, but not the other drugs, reduced noradrenaline levels in cortex (62%, $P < 0.001$) and hippocampus (53%, $P < 0.01$) and also, but to a lesser extent, dopamine levels in striatum (16%, $P > 0.05$) (data not shown).

In naive rats, clonidine (1 mg/kg and 3 mg/kg, i.p.) also decreased the synthesis of 5-HTP in the cerebral cortex (41% and 42%) and hippocampus (29% and 61%) (Fig. 4). However, the decrease induced by clonidine on 5-HTP synthesis in naive rats was abolished in rats pretreated with EEDQ (Fig. 4). Moxonidine (1 mg/kg and 10 mg/kg, i.p.) also induced a decrease in the synthesis of 5-HTP in the cerebral cortex (28% and 81%) and hippocampus (50% and 74%) which was not observed in EEDQ-pretreated rats (Fig. 4). Rilmenidine (10 mg/kg, i.p.) modestly decreased the synthesis of 5-HTP in the cerebral cortex (27%), but not in the hippocampus, and the effect was abolished by EEDQ (Fig. 4). The compound AGN-192403 (5 mg/kg and 10 mg/kg, i.p.) did not modify the

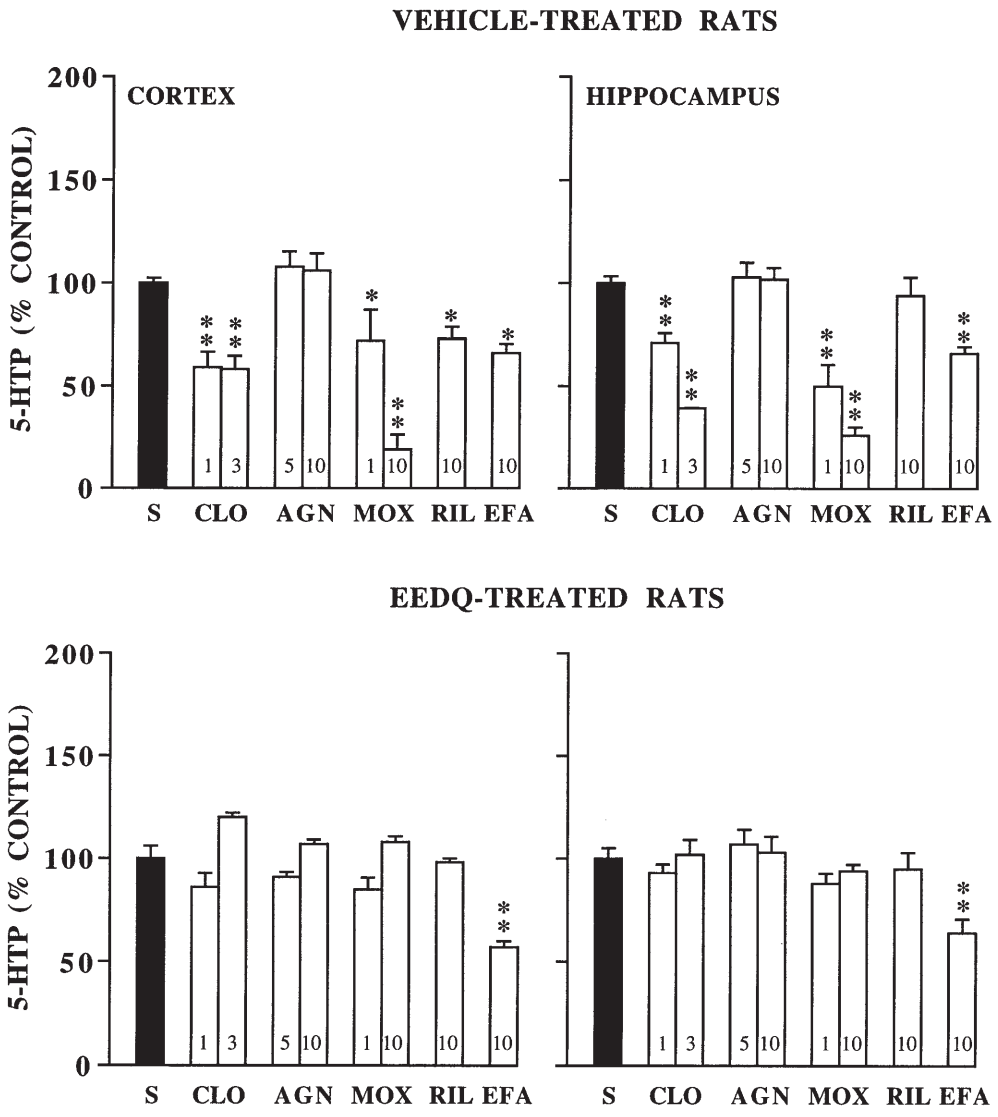

Fig. 4 Acute effects (1 h) of several I_1-imidazoline drugs on 5-HTP accumulation in the cerebral cortex and hippocampus of naive rats and rats pretreated for 5 h with EEDQ (1.6 mg/kg). Other details as in Fig. 3. [Mean control values in ng/g 30 min in naive rats: 5-HTP 107 ± 3, n = 12 (cortex) and 179 ± 6, n = 13 (hippocampus); in rats pretreated with EEDQ: 5-HTP 138 ± 8, n = 7 (cortex) and 182 ± 9, n = 6 (hippocampus)]. *Bars* represent means ± SEM derived from 3–7 experiments for each drug as percentage of saline-treated controls. One-way ANOVA followed by Scheffé or Fisher LSD (least statistical deviation) were used for statistical evaluation. *P < 0.01; **P < 0.001 when compared with the saline control group

synthesis of 5-HTP in any brain region in naive or EEDQ-pretreated rats (Fig. 4).

The acute administration of efaroxan (10 mg/kg, i.p.) decreased the synthesis of 5-HTP in the cerebral cortex (34%) and hippocampus (34%) (Fig. 4). After EEDQ, a similar inhibitory effect of efaroxan on 5-HTP formation was obtained in the cerebral cortex (43%) and hippocampus (36%) (Fig. 4). The inhibitory effect of efaroxan on 5-HTP synthesis (cortex and hippocampus) was mediated by 5-HT_{1A} autoreceptors, because in both brain regions it was antagonized by the selective 5-HT_{1A} antagonist WAY 100135 (10 mg/kg, i.p.) (Fig. 5). As expected, the stimulatory effect of efaroxan on dopa synthesis (cortex and hippocampus) was not antagonized by WAY 100135 (Fig. 5). In the striatum, however, efaroxan decreased the synthesis of dopa, which was unaffected by EEDQ (see Fig. 3), and this effect was antagonized by WAY 100135, suggesting the involvement of inhibitory 5-HT_{1A} heteroreceptors (dopa accumulation in rats treated with WAY 100135 (10 mg/kg): 1913 ± 181 ng/g 30 min (n = 5); dopa accumulation in rats treated with efaroxan (10 mg/kg): 813 ± 77 ng/g 30 min (n = 3), P < 0.001; dopa accumulation in rats treated with efaroxan after pretreatment with WAY 100135: 1499 ± 43 ng/g 30 min (n = 3).

Effects of I_2-imidazoline ligands on brain tyrosine and tryptophan hydroxylase activity in vivo

In naive rats, the acute treatment with idazoxan (mixed I_2/α_2 antagonist; 20 mg/kg, i.p.) markedly increased dopa accumulation in the cerebral cortex (111%) and hippocampus (87%) but it reduced dopa synthesis in the striatum (30%). In EEDQ-pretreated rats the effects of idazoxan on dopa synthesis were abolished (Fig. 6). Idazoxan reduced noradrenaline levels in the cortex (67%, P < 0.001) and hippocampus (64%, P < 0.001).

The selective I_2-imidazoline drug 2-BFI (20 mg/kg, i.p.) did not alter the synthesis of dopa in the cerebral cortex but it caused a modest nonsignificant decrease in hippocampus (23%) and a marked decrease in striatum (75%) (Fig. 6). In EEDQ-pretreated rats, 2-BFI (20 mg/kg, i.p.) significantly decreased dopa accumulation in the

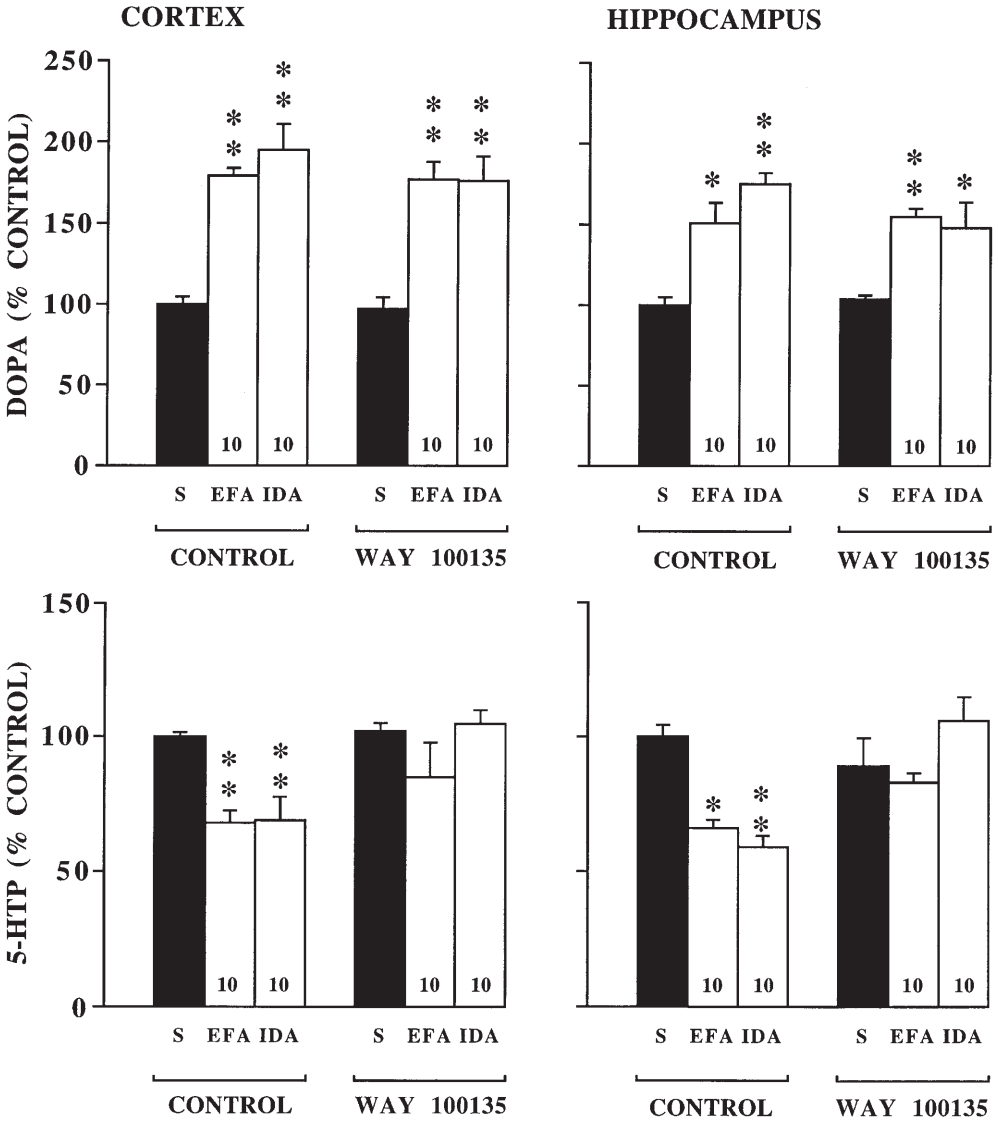

Fig. 5 Effects of the selective 5-HT_{1A} receptor antagonist WAY 100135 on efaroxan- and idazoxan-induced changes in dopa and 5-HTP synthesis in rat brain. The drugs (10 mg/kg) were injected i.p. alone or in combination 5 min apart (WAY 100135 followed by efaroxan or idazoxan). Other details as in Fig. 3. [Mean control values in ng/g 30 min in naive rats: dopa 88 ± 4, $n = 9$ (cortex) and 90 ± 5, $n = 9$ (hippocampus); 5-HTP 104 ± 2, $n = 9$ (cortex) and 179 ± 8, $n = 9$ (hippocampus)]. *Bars* represent means ± SEM derived from 3–5 experiments for each drug as percentage of saline-treated controls. *$P <$ 0.01; **$P < 0.001$ when compared with the corresponding control (Student's *t*-test)

cerebral cortex (26%) and hippocampus (44%). In the striatum, the strong inhibitory effect of 2-BFI on dopa/dopamine synthesis was not modified by pretreatment with EEDQ (76%) (Fig. 6). 2-BFI reduced noradrenaline levels in the cerebral cortex (58%, $P < 0.001$) and hippocampus (57%, $P < 0.001$) and it also decreased dopamine levels in striatum (36%, $P < 0.01$). In contrast, LSL 61122 (10 mg/kg, i.p.), another selective I_2-imidazoline ligand, did not alter the synthesis of dopa in any brain region in naive or EEDQ-pretreated rats (Fig. 6).

The acute treatment with idazoxan (20 mg/kg, i.p.) decreased 5-HTP formation in both the cerebral cortex (12%) and hippocampus (30%). In EEDQ-pretreated rats idazoxan also decreased 5-HTP accumulation in the cerebral cortex (26%) but not in the hippocampus (Fig. 7). Similarly to efaroxan, the inhibitory effect of idazoxan on 5-HTP synthesis (cortex and hippocampus) was mediated by activation of 5-HT_{1A} autoreceptors, because in both brain regions it was antagonized by WAY 100135 (Fig. 5). The stimulatory effect of idazoxan on dopa synthesis was not antagonized by WAY 100135 (Fig. 5).

The acute treatments with 2-BFI (20 mg/kg, i.p.) and LSL 61122 (10 mg/kg, i.p.) did not alter 5-HTP accumulation in any brain region of naive or EEDQ-pretreated rats (Fig. 7).

Effects of 2-BFI on dopa/dopamine synthesis and metabolite levels in rat striatum

2-BFI, a highly selective and potent I_2-imidazoline drug, markedly decreased dopa synthesis in the striatum of naive and EEDQ-treated rats as well as in the cerebral cortex and hippocampus of EEDQ-treated rats (Fig. 6). To investigate the mechanism involved in the inhibitory effect of 2-BFI on dopa/dopamine synthesis, further experiments were performed in the striatum of naive rats. The acute treatment with 2-BFI (1, 3, 7, 10 and 20 mg/kg, 1 h) induced a clear dose-dependent inhibitory effect on dopa accumulation in the striatum (4%–75%, ED_{50}: 5.9 ± 0.7 mg/kg) (Fig. 8). In this brain region, 2-BFI also reduced in a dose-dependent manner dopamine levels (6%–36%) and dose-dependently increased the levels of DOPAC (15%–

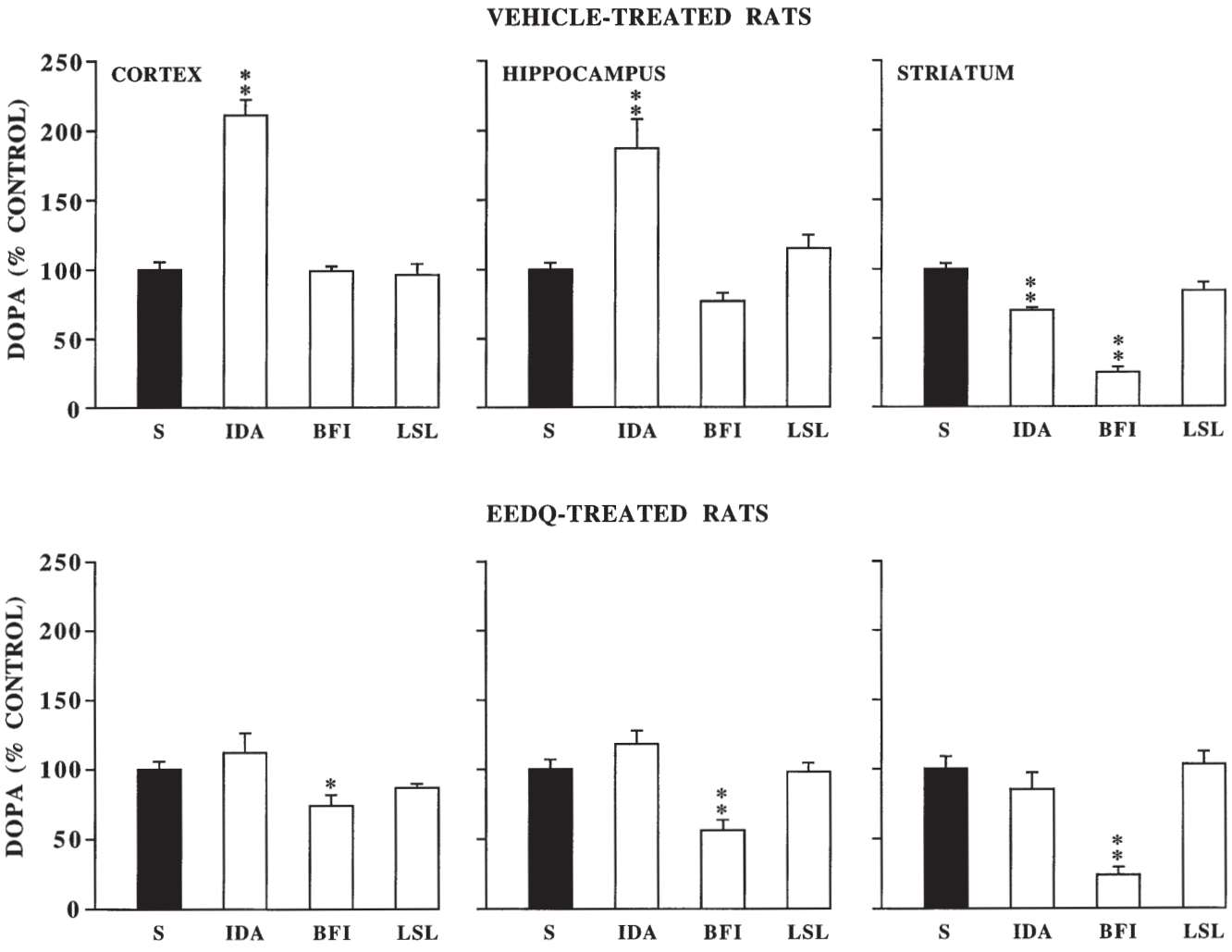

Fig. 6 Acute effects (1 h) of several I_2-imidazoline drugs on dopa accumulation in the cerebral cortex, hippocampus and striatum of naive rats and rats pretreated for 5 h with EEDQ (1.6 mg/kg). Idazoxan (*IDA*; 20 mg/kg), 2-BFI (*BFI*; 20 mg/kg), LSL 61122 (*LSL*; 10 mg/kg). Other details as in Fig. 3. [Mean control values in ng/g 30 min in naive rats: dopa 89 ± 5, n = 13 (cortex), 86 ± 4, n = 14 (hippocampus) and 1562 ± 67, n = 15 (striatum); in rats pretreated with EEDQ: dopa 170 ± 10, n = 8 (cortex), 157 ± 11, n = 6 (hippocampus) and 3005 ± 268, n = 8 (striatum)]. *Bars* represent means ± SEM derived from 3–5 experiments for each drug as percentage of saline-treated controls. One-way ANOVA followed by Scheffé or Fisher LSD (least statistical deviation) were used for statistical evaluation. *$P < 0.05$; **$P < 0.001$ when compared with the saline control group

95%) and HVA (24%–74%), the main metabolites of dopamine (Fig. 8).

2-BFI has been shown to inhibit at high concentrations MAO enzymes in vitro (see Ozaita et al. 1997 and other references therein). However, the current results were not those expected for an inhibition of MAO by 2-BFI in vivo (i.e. decreased dopamine levels and increased levels of its metabolites). For comparison, phenelzine (30 mg/kg, i.p., 1 h), a prototypical inhibitor of MAO, also reduced the synthesis of dopa (40%) but, as expected, it increased the levels of dopamine (59%) and decreased those of its metabolites DOPAC and HVA to undetectable levels (Fig. 8). These results indicated that 2-BFI does not behave as an inhibitor of MAO in vivo (at least in the range of doses used in the current study) and suggested that the inhibitory effect of this drug on dopa synthesis in the striatum and other brain regions (see Fig. 6) could be mediated through an interaction with I_2-imidazoline receptors.

To assess this possibility, imidazoline receptors were irreversibly inactivated with the alkylating agent IBI (Boronat et al. 1998) or blocked with the antagonist KU-14R (Chan et al. 1997) and then the inhibitory effect of 2-BFI on dopa/dopamine synthesis in the striatum re-tested. IBI (60 mg/kg, i.p., 6 h) alone did not modify dopa accumulation in the striatum (Fig. 9). In rats pretreated with IBI (60 mg/kg for 6 h), the inhibitory effect of 2-BFI (7 mg/kg, i.p.) on dopa accumulation remained unchanged (57%) (Fig. 9). Similarly, KU-14R (7 mg/kg and 20 mg/kg, i.p., 90 min) alone did not alter dopa accumulation in the striatum, and the inhibitory effect of 2-BFI (7 mg/kg, i.p.) remained unchanged after KU-14R pretreatment (44% and 47%) (Fig. 9). Together, these results suggested that the inhibitory effect of 2-BFI on dopa/dopamine synthesis in rat striatum is not mediated by an interaction with imidazoline receptors.

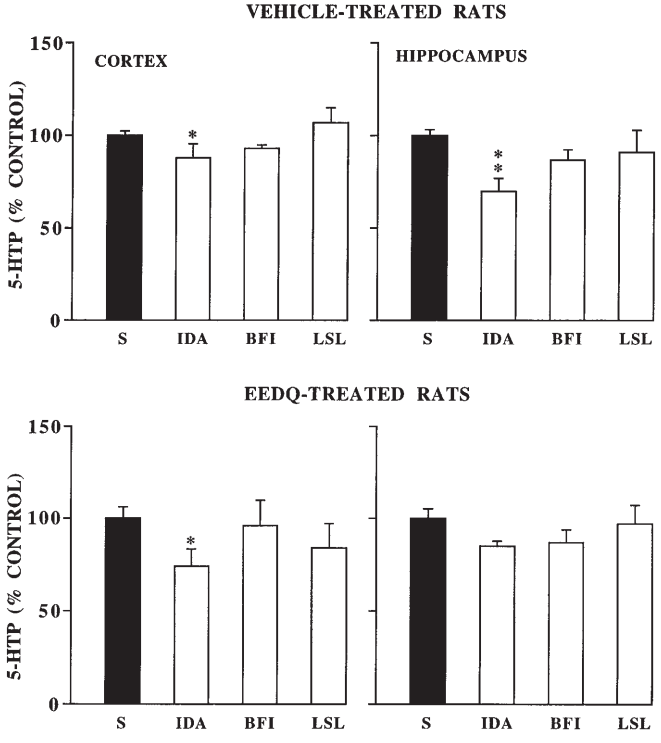

Fig. 7 Acute effects (1 h) of several I_2-imidazoline drugs on 5-HTP accumulation in the cerebral cortex and hippocampus of naive rats and rats pretreated for 5 h with EEDQ (1.6 mg/kg). Other details as in Figs. 2 and 4. [Mean control values in ng/g 30 min in naive rats: 5-HTP 107 ± 3, *n* = 12 (cortex) and 179 ± 6, *n* = 13 (hippocampus); in rats pretreated with EEDQ: 5-HTP 138 ± 8, *n* = 7 (cortex) and 182 ± 9, *n* = 6 (hippocampus)]. *Bars* represent means ± SEM derived from 3–5 experiments for each drug as percentage of saline-treated controls. One-way ANOVA followed by Scheffé or Fisher LSD (least statistical deviation) were used for statistical evaluation. **P* < 0.05; ***P* < 0.01 when compared with the saline control group

Similarly, the inhibitory effect of 2-BFI (7 mg/kg, i.p.) on dopa/dopamine synthesis in rat striatum was not antagonized by the selective 5-HT_{1A} antagonist WAY100135 (10 mg/kg, i.p.) or by the specific NMDA receptor antagonist dizocilpine (0.1 mg/kg, i.p.) (data not shown), suggesting that these receptors are not involved in the effect of 2-BFI. Moreover, 2-BFI displayed very low affinity for dopamine D_2 receptors (competition against [^{3}H]raclopride binding in rat striatum, K_i: 47 µmol/l; data not shown) and therefore it is also very unlikely that its inhibitory effect on dopa/dopamine in the striatum was mediated through activation of presynaptic dopamine D_2-autoreceptors.

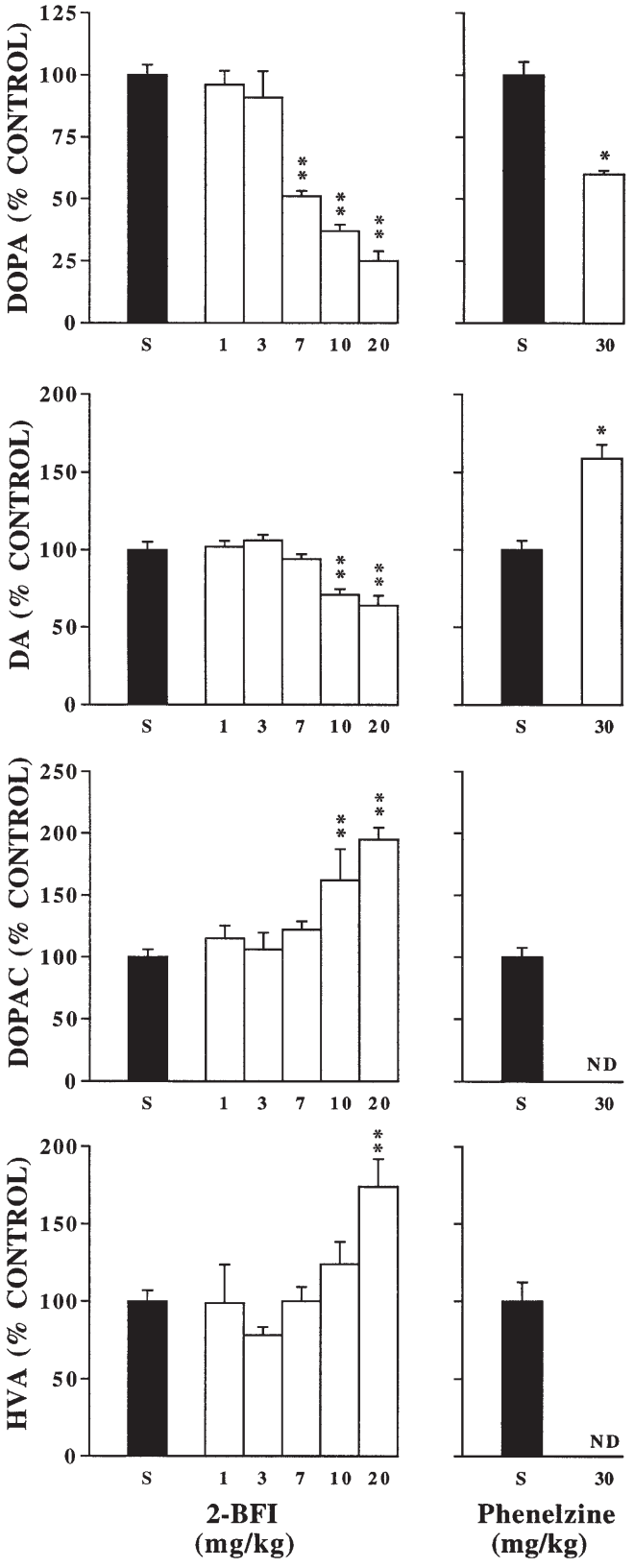

Fig. 8 Effects of various doses of 2-BFI (1–20 mg/kg) and phenelzine (30 mg/kg) on dopa synthesis and levels of dopamine, DOPAC and HVA in the rat striatum. [Mean control values in ng/g 30 min: dopa 1598 ± 65, *n* = 10; dopamine 8114 ± 418, *n* = 11; DOPAC 308 ± 19, *n* = 9; HVA 570 ± 40, *n* = 12] (*ND* levels not detected). *Bars* represent means ± SEM derived from 3–7 experiments (2-BFI) and from 3 experiments (phenelzine) as percentage of saline-treated controls. **P* < 0.01; ***P* < 0.001 when compared with the corresponding control (one-way ANOVA followed by Scheffé's test or Student's *t*-test)

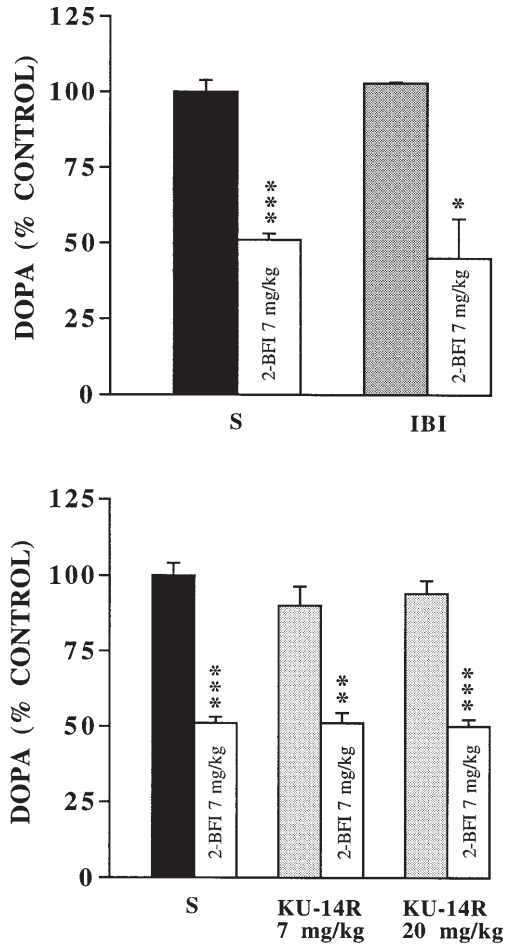

Fig. 9 Effects of 2-BFI (7 mg/kg) on dopa accumulation in the rat striatum after pretreatment of rats with IBI (60 mg/kg, 5 h) or KU-14R (7 mg/kg and 20 mg/kg, 30 min). [Mean control values in ng/g 30 min: dopa 1598 ± 65, $n = 10$ (naive rats)]. *Bars* represent means ± SEM derived from 3–4 experiments as percentage of corresponding control. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ when compared with the corresponding control (Student's *t*-test)

Discussion

Various types of imidazoline receptors (I_1-, I_2- and I_3-sites) have been characterized and some functions associated with these sites have been defined (for a review see Eglen et al. 1998). Recently, imidazoline receptor proteins have been localized in axon terminals of the rat CNS (Ruggiero et al. 1998), suggesting a possible role for these novel receptors in neurotransmitter release and synthesis in the brain. This study was designed to characterize the in vivo effects of various imidazoline drugs on monoamine (noradrenaline, dopamine and 5-HT) synthesis through interaction with putative presynaptic imidazoline receptors in the cerebral cortex, hippocampus and striatum of rats. The results demonstrate that most imidazoline drugs (mixed imidazoline receptor/α_2-adrenoceptor ligands) modulated the synthesis of monoamines in the brain through an interaction with α_2-adrenoceptors or 5-HT_{1A} receptors (i.e. the effects of the drugs were abolished after blockade of these receptors). The selective I_2-imidazoline drug 2-BFI induced a potent inhibitory effect on dopa/dopamine synthesis in the striatum through an unknown mechanism not related to I_2-sites. Therefore, the current results do not provide functional evidence for the existence of presynaptic imidazoline receptors regulating the synthesis of monoamines in the rat brain.

The functional characterization of receptors was assessed by monitoring the synthesis of dopa and 5-HTP in vivo in naive rats and in rats pretreated with EEDQ, an irreversible α_2-adrenoceptor antagonist with little effect on I_2-imidazoline receptors (Miralles et al. 1993b). EEDQ has been used as a useful tool to investigate the in vivo effects of imidazoline receptor ligands on the activity of locus coeruleus noradrenergic neurones (Pineda et al. 1993) and also on the release of noradrenaline in the brain (Meana et al. 1997). The inactivation of α_2-adrenoceptors by EEDQ resulted in a time-dependent increase in the synthesis of dopa in the cerebral cortex, hippocampus and striatum, demonstrating clearly the existence of a powerful inhibitory tone of α_2-autoreceptors (and perhaps of α_2-heteroreceptors on dopaminergic terminals) on tyrosine hydroxylase activity (Esteban et al. 1996). The maximal stimulatory effect of EEDQ on dopa synthesis was already observed with a low dose of the alkylating agent (1.6 mg/kg, 6 h) which almost eliminates (> 90%) α_2-adrenoceptor radioligand binding (Meller et al. 1988; Miralles et al. 1993b). In contrast, EEDQ treatment did not induce significant increases in 5-HTP synthesis in any brain region, suggesting that α_2-heteroreceptors located on serotonergic terminal axons receive little physiological tone. On the other hand, EEDQ treatment also reduced the content of brain noradrenaline and dopamine, but not that of 5-HT, an effect probably caused by a pronounced increase in the release of the two catecholamines subsequent to blockade/inactivation of α_2-adrenoceptors (see Pi and García-Sevilla 1992).

Effects of I_1-imidazoline ligands on brain monoamine synthesis

The in vivo modulation of monoamine synthesis through presynaptic I_1-imidazoline receptors in the rat brain is unlikely. Most of the effects induced by the I_1-imidazoline ligands tested were abolished after irreversible inactivation of α_2-adrenoceptors with EEDQ, suggesting that these latter receptors were responsible for the observed drug effects. In brain tissues, clonidine, moxonidine and rilmenidine show high affinity for I_1-sites (K_i values: 1–6 nmol/l) and also for α_2-adrenoceptors (K_i values: 4–180 nmol/l) (Ernsberger et al. 1993, 1997) and they possess low affinity for I_2-sites (K_i values: 950–1500 nmol/l) (Miralles et al. 1993a). The I_1/α_2 affinity ratio for moxonidine and rilmenidine is higher than that of clonidine. In naive rats, however, whereas clonidine (1 mg/kg) and moxonidine (1 mg/kg) markedly decreased brain dopa and 5-HTP synthesis, a high dose of rilmenidine (10 mg/kg) only slightly decreased dopa synthesis in the striatum and

5-HTP synthesis in the cerebral cortex. In addition, the effects displayed by clonidine, moxonidine and rilmenidine on monoamine synthesis in naive rats clearly correlated with the affinities that these drugs possess for α_2-adrenoceptors (Ernsberger et al. 1993, 1997). Moreover, the selective I_1-imidazoline ligand AGN-192403 (K_i value: 42 nmol/l), a compound with very low affinity for α_2-adrenoceptors (K_i value > 20 μmol/l) (Munk et al. 1996), did not alter the synthesis of dopa or 5-HTP in any brain region of naive rats or rats pretreated with EEDQ, a negative result in line with the above findings.

The effects of the mixed I_1/α_2 antagonist efaroxan (Ernsberger et al. 1997; Szabo and Urban 1997) on dopa/noradrenaline synthesis in the cerebral cortex and hippocampus were most probably mediated through blockade of α_2-adrenoceptors (i.e. effects abolished by EEDQ), and those on 5-HTP/5-HT synthesis through activation of 5-HT_{1A} receptors (i.e. effects antagonized by WAY100135). Several α_2-adrenoceptor antagonists possess affinity for 5-HT_{1A} receptors and behave as specific 5-HT_{1A} agonists in vivo (Esteban et al. 1996; Lladó et al. 1996). Thus, the stimulation of 5-HT_{1A} autoreceptors has been shown to decrease the synthesis of 5-HTP in brain (Fernstrom et al. 1990; Lladó et al. 1996). It is of interest to note that pretreatment of rats with the selective 5-HT_{1A} receptor antagonist WAY100135 antagonized the inhibitory effect of efaroxan and idazoxan on 5-HTP synthesis in the cerebral cortex and hippocampus, but it did not prevent the stimulatory effect on dopa synthesis in the same brain regions. Moreover, the inhibitory effect of efaroxan on dopa/dopamine synthesis in the striatum (not abolished by EEDQ) was related to activation of 5-HT_{1A} heteroreceptors, because it was antagonized by WAY 100135. The 5-HT_{1A} heteroreceptors located on dopaminergic nerve terminals in striatum have been shown to mediate an inhibition of tyrosine hydroxylase activity (Johnson et al. 1993). The interaction of efaroxan and idazoxan with 5-HT_{1A} autoreceptors (inhibitory effect on 5-HTP synthesis) and α_2-heteroreceptors (possible stimulatory effect on 5-HTP synthesis) may also explain why the effect of a 10-mg/kg dose on 5-HTP synthesis was more pronounced than that of a 20-mg/kg dose (see drug effects in Figs. 5, 7; vehicle-treated rats; see also Lladó et al. 1996).

Effects of I_2-imidazoline ligands on brain monoamine synthesis

The in vivo modulation of monoamine synthesis through presynaptic I_2-imidazoline receptors in the rat brain is also unlikely. The effects of idazoxan, a mixed I_2/α_2 antagonist (Ernsberger et al. 1997), were very similar to those of efaroxan, suggesting the involvement of α_2-adrenoceptors for the effects on dopa synthesis (i.e. effects abolished by EEDQ) and 5-HT_{1A} autoreceptors for the effects on 5-HTP synthesis (i.e. effects antagonized by WAY 100135), because idazoxan has been shown to behave as a potent 5-HT_{1A} receptor agonist in vivo (Lladó et al. 1996).

In EEDQ-treated rats, the selective I_2-ligand 2-BFI (K_i value: 3.8 nmol/l; Ozaita et al. 1997) induced strong inhibitory effects on dopa synthesis in the cerebral cortex, hippocampus and striatum. Because the striatum is a brain region enriched with a high density of I_2-imidazoline receptors, the effect of 2-BFI was re-assessed in this region after partial alkylation of I_2-sites with IBI (60 mg/kg, 6 h; reduction of I_2-sites: 41%) (Boronat et al. 1998). Under these experimental conditions, however, the strong inhibitory effect of 2-BFI on dopa synthesis in the striatum remained unchanged, suggesting a mechanism not related to I_2-sites. Moreover, the compound LSL 61122 which also possesses high affinity for I_2-sites (K_i value: 0.1 nmol/l; Ozaita et al. 1997) did not modify the synthesis of dopa or 5-HTP in any brain region in naive or EEDQ-pretreated rats. In addition, the imidazoline receptor antagonist KU-14R (Chan et al. 1997) also was unable to prevent the potent inhibitory effect of 2-BFI on dopa synthesis in the striatum. Therefore, the possible involvement of imidazoline receptors in the effect of 2-BFI was discounted. Similar experiments with the antagonists WAY100135 and dizocilpine also discarded the involvement of 5-HT_{1A} or NMDA receptors in the effect of 2-BFI in rat striatum.

2-BFI and LSL61122 have been shown to inhibit MAO-A (IC_{50} values: 11 μmol/l and 100 μmol/l, respectively) and MAO-B (IC_{50} values: 23 μmol/l and 32 μmol/l, respectively) enzymes in vitro (Ozaita et al. 1997). Also, 2-BFI was shown to increase the extracellular levels of noradrenaline in cerebral cortex of rats (Lalies and Nutt 1995), which was interpreted as a result of inhibition of MAO. However, the current results do not agree with this interpretation. In fact, 2-BFI reduced the content of dopamine and increased those of its metabolites DOPAC and HVA in the striatum. These effects are the opposite to those expected for an inhibition of MAO-B (see for comparison the effects of phenelzine; Fig. 8), indicating that 2-BFI does not behave as an inhibitor of MAO in vivo. Moreover, LSL 61122, which also shows a similar μmol/l potency for the inhibition of MAO-B (see above), did not modify the synthesis of dopa or the content of dopamine and its metabolites in the striatum. Because 2-BFI also decreased the content of noradrenaline in the cerebral cortex and hippocampus, it is possible that this drug could behave as a releasing agent of monoamines in the brain. This possibility will require further investigation.

In conclusion, the results of this study do not provide functional evidence for the existence of presynaptic imidazoline receptors regulating the synthesis of monoamines in the rat brain in vivo.

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