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Anxiolytic homophthalazines increase Fos-like immunoreactivity in selected brain areas of the rat

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Abstract

Nerisopam, an anxiolytic and antipsychotic homophthalazine induces rapid, intense expression of Fos-like immunoreactivity in the rostral, dorsomedial and lateral parts of the striatum in the rat. Fos-positive cells also occurred in the globus pallidus, the olfactory tubercle and in the accumbens nucleus (in the cone and shell portions) but the substantia nigra, the entopeduncular and the subthalamic nuclei were virtually Fos-negative. 5 h after nerisopam application, however, cells in the reticular zone of the substantia nigra showed Fos-like immunopositivity. After a daily application of nerisopam for two weeks, relatively weak Fos-like immunoreactivity was observed in the striatum and the subthalamic nucleus but not in the globus pallidus. Unilateral surgical transection of the striato–nigral pathway, which depleted tyrosine hydroxylase immunostaining in the ipsilateral striatum did not influence nerisopam-induced Fos-like immunoreactivity in the striatal neurons, either ipsi- or contralateral to the knife cut. Our results suggest that the striatal neurons are the primary targets of this anxiolytic and antipsychotic drug in the central nervous system. © 1997 Elsevier Science B.V.

Keywords: Homophthalazine; 2,3-benzodiazepine; Anxiolytic drug; Antipsychotic drug; c-*fos* expression; Striatum

1. Introduction

Nerisopam (GYKI-52322, EGIS-6775) is a homophthalazine (2,3-benzodiazepine) derivative with strong anxiolytic and antipsychotic potencies (Andrási et al., 1987; Horváth et al., 1989). Its anxiolytic activity is stronger than that of the other members of the homophthalazine family (Horváth et al., 1992b). Although homophtalazines are structurally related to the 'classical' 1,4-benzodiazepines, they have different chemical and pharmacological entities. By autoradiography, nerisopam, like other homophthalazines, shows a specific distribution pattern of binding sites exclusively in the components of the striato–pallidal and striato–nigral systems (Horváth et al., 1993, 1994). The very high labeling in the caudate-putamen, the globus pallidus and the substantia nigra, and no binding in the cerebellum, the cerebral cortex, the thalamus or brainstem areas clearly indicate that the binding site of homophthalazines differs from that of the 1,4-benzodiazepine receptors (Horváth et al., 1992a). The mechanism of action of nerisopam on the central nervous system is still unknown. Former in vitro studies indicate that homophthalazines do not compete for dopamine or serotonin receptors, or for γ-aminobutyric acid (GABA), kainate or diazepam binding sites in the brain (Andrási et al., 1987; Horváth et al., 1989).

The immediate-early gene c-*fos* has been reported to be a marker of neurons that are activated by various types of stimuli. Histochemical demonstration of the expression of c-*fos*, or its protein product Fos, have provided a useful tool to visualize and localize metabolically activated neurons in the central nervous system (Morgan and Curran, 1991). This technique has been widely used to investigate the effect of dopaminergic agents, typical and atypical antipsychotic drugs on the activity of striatal neurons (see Wirtshafter and Asin, 1994). Fos immunohistochemistry has been designed in the present experiment to localize the

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Table 1
Spontaneous locomotor activity

Experimental groups	Motility counts				
	0–5′	6–10′	11–15′	16–20′	Total
Control (vehicle-treated)	381	373	352	436	1542
30 mg/kg nerisopam	225	151	106	110	592
30 mg/kg nerisopam + nigro–striatal transection	268	107	102	39	516

3 animals in each group.

primary target sites of nerisopam in the central nervous system.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (Charles River, Gödöllő, Hungary), weighing 275–300 g, were used in this study. The animals were housed four per cage in a room that was maintained under constant temperature (22 ± 1°C) and humidity on a 12 h light–dark cycle (light on 06.00 a.m.). They received regular rat chow (dry pellets) and tap water ad libitum. Food uptake was restricted 18 h before the experiments.

2.2. Drugs

Nerisopam (1-[4-aminophenyl]4-methyl-7,8-dimethoxy-5*H*-2,3-benzodiazepine) was synthesized as described by Láng et al. (1985). Antiserum for c-*fos* (rabbit polyclonal immunoglobulin G [IgG]) was purchased from Oncogene Science (New York, NY, USA), biotinylated anti-rabbit IgG and avidin–biotinylated–peroxidase complex from Vector (Burlingame, CA, USA), monoclonal antiserum for tyrosine hydroxylase from Incstar (Stillwater, MN, USA), normal goat serum from Dakopatts (Glostrup, Denmark).

2.3. Experimental protocol

The effect of nerisopam on c-*fos* expression in various brain areas was investigated in three experiments (12–14 animals per experiment). At the end of the procedures, animals were lightly anesthetized with ether and perfused intracardially with a fixative solution (4% paraformaldehyde, 0.19% picric acid in 0.1 M phosphate buffer, pH 7.35), postfixed in the same solution for 2 h, then cryoprotected in 20% sucrose in 0.1 M phosphate buffer for 24 h.

2.3.1. Single dose of nerisopam

The animals received 30 mg/kg nerisopam per os. Nerisopam was suspended in 2% Tween 80, diluted in distilled water and given through a gastric cannula. A group of rats were perfused 90 min, an other one 5 h after the administration. Each group had vehicle-treated controls.

2.3.2. Chronic nerisopam administration

Rats in this group received 20 mg/kg nerisopam (as well as vehiculum) per os daily for 13 days. The drug was fresh prepared (see Section 2.3.1) daily. 90 min after the last administration, the animals were perfused.

2.3.3. Unilateral hemisection of the striato–nigral pathway

The head of the animals was fixed in a stereotaxic device (David Kopf, CA, USA) in a 5° 'nose-down' position. One side, 4.5 mm caudal to the level of the bregma, a 3.0 mm long line-shaped coronal hole was opened on the skull with a drill 1.0 to 4.0 mm lateral to the midline. By a vertical penetration of a 3.0 mm wide 'glass knife' cut out from a histological coverslip (Palkovits et al., 1982) down to the ventral surface of the brain, the striato–nigral connections were completely transected, unilaterally. Sham-operated rats were treated in a same way, but the knife was lowered not deeper than the corpus callosum. Thirteen days after surgery, rats received a single dose of 30 mg/kg nerisopam per os, and sacrificed 90 min after the application. Brains were removed, frozen on dry ice and sliced for Fos immunohistochemistry, as well as for the histological control of the surgery.

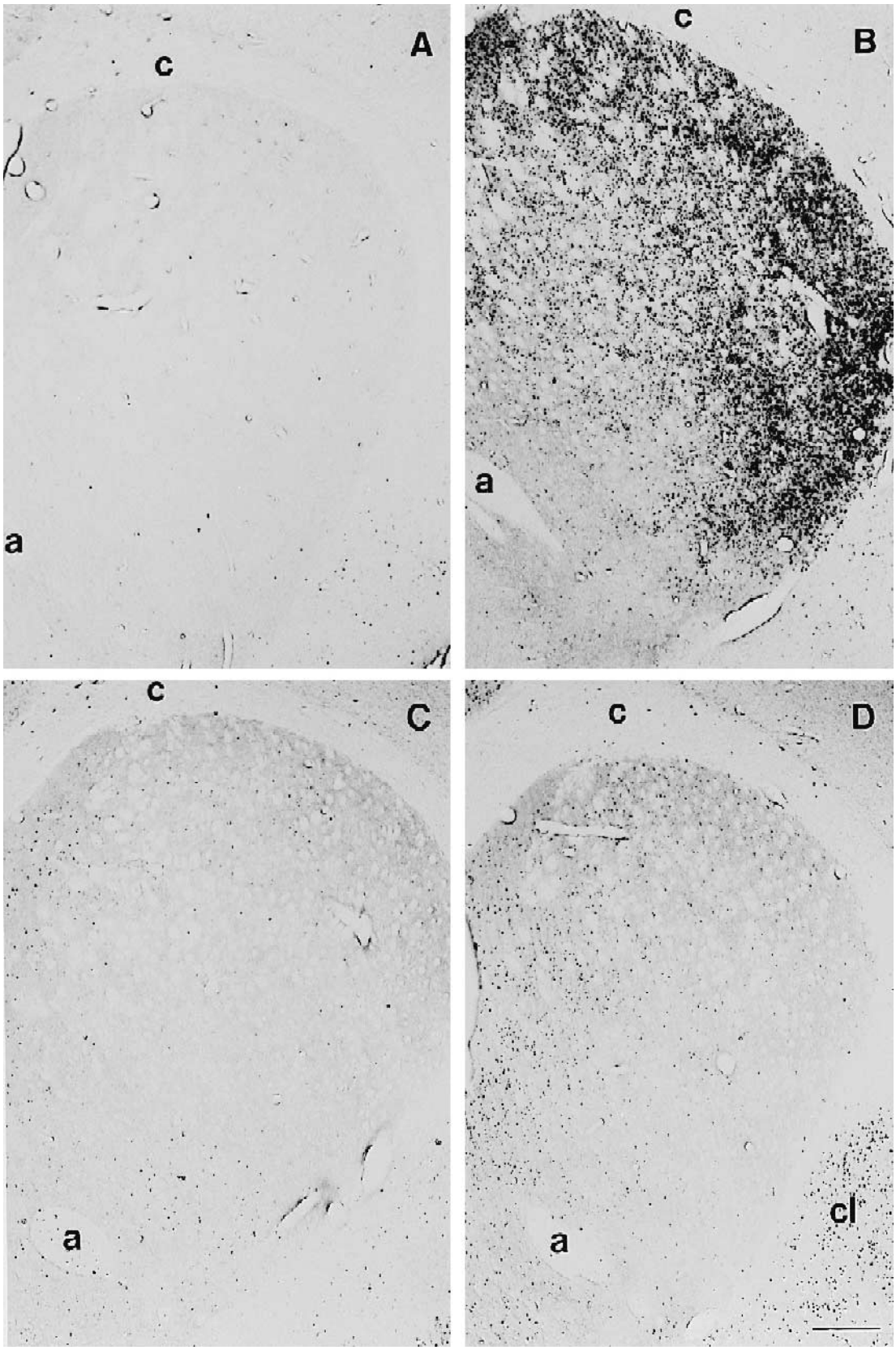
2.4. Monitoring of the spontaneous locomotor activity

After 90 min of nerisopam application, the motility of the animals (3 rats per group) was recorded for 20 min by Animex-type capacitance motimeters (home-built).

2.5. c-fos immunohistochemistry

Serial, coronal sections of 50 μm thickness were cut through the forebrain and the mesencephalon with a freez-

Fig. 1. Fos-like immunoreactive neurons in the rostral part of the caudate-putamen (head of the caudate nucleus). Coronal sections 1.0–1.1 mm rostral to the level of the bregma. (A) No Fos-like immunopositivity in the control animals. (B) High density (especially in the dorsomedial and the ventrolateral portions of the caudate-putamen) of Fos-positive cells 90 min after nerisopam administration. (C) Slight Fos-like activity 5 h after the drug treatment. (D) Daily treatment of nerisopam for 13 consecutive days resulted in light intensity and low density of Fos-immunopositive cells in the caudate-putamen. Abbreviations: a, anterior commissure; c, corpus callosum; cl, claustrum. Bar scale: 500 μm.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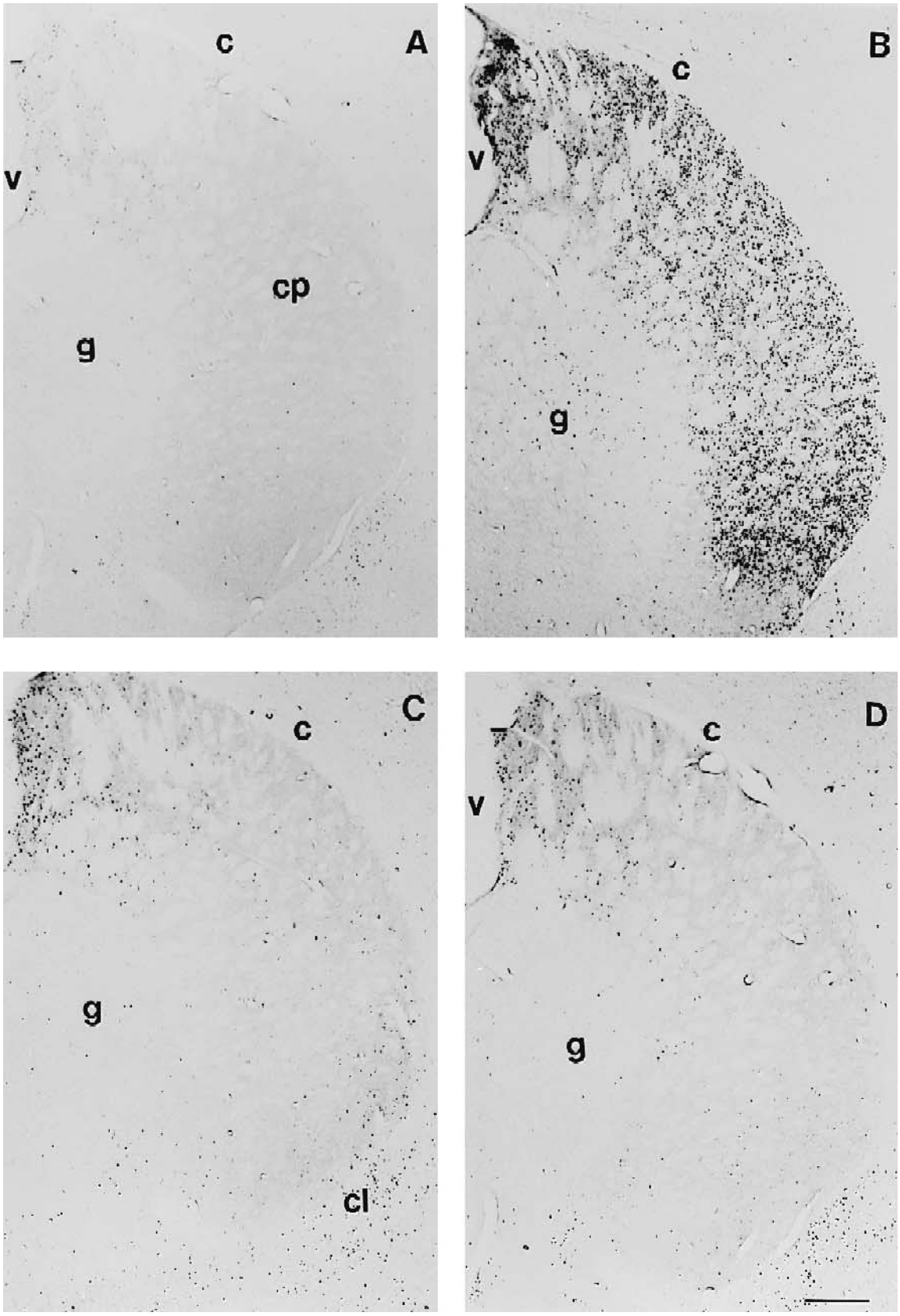
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ing microtome (Frigomobil, Reichert-Jung) at −20°C. The sections were washed in 0.1 M phosphate buffer, incubated in 3% H_2O_2 for 10 min, rinsed again in phosphate buffer, and incubated in 0.5% Triton X-100 overnight at 4°C. After rinsing three times in phosphate buffer, sections were placed in 10% normal goat serum for 1 h at room temperature. Then, sections were incubated in c-*fos* antiserum (in 1:1000 dilution) for 48 h at 4°C. Biotinylated anti-rabbit IgG was applied as a second antibody at a dilution of 1:500 for 1 h at room temperature. Subsequently, sections were treated with avidin–biotinylated–peroxidase complex for 1 h. The Fos-like immunoreactivity was revealed by the nickel–diaminobenzidine method (600 mg nickel–ammonium sulphate, 50 mg diaminobenzidine, 100 μl 3% H_2O_2 in 100 ml 0.05 M Tris–HCl buffer, pH 7.4). After washing in Tris–HCl buffer, brain slices were mounted onto gelatine coated slides, dried and coverslipped with depex. The Fos-like immunoreactivity appeared as bluish–black dots in the cell nuclei. The control incubated without the c-*fos* antibody.

One set of the sections was used for double-staining for c-*fos* and tyrosine hydroxylase. Immediately after the development of Fos immunoreactivity, sections were rinsed in 0.1 M phosphate buffer and incubated in monoclonal mouse antiserum for tyrosine hydroxylase at a dilution of 1:1000 for 48 h at 4°C. The tyrosine hydroxylase antibody was developed by diaminobenzidine without nickel intensification. The tyrosine hydroxylase immunoreactivity gave brown labeling in the cytoplasm of the neurons with or without the bluish–black Fos-positive cell nuclei.

3. Results

3.1. Spontaneous locomotor activity

Nerisopam evoked a strong sedative, motility reducing effect. In nerisopam-treated rats, the motility counts/20 min was reduced, while there was no significant difference between nerisopam-treated operated (striato–nigral transection) and sham-operated groups (Table 1).

3.2. Fos-immunohistochemistry

Very few if any of the cell nuclei with Fos-like immunoreactivity were found in the striatum (Fig. 1A, Fig. 2A, Fig. 3A), the pallidum (Fig. 2A, Fig. 3A), the substantia nigra (Fig. 4A) and in the entopeduncular nucleus (Fig. 5A) in control (vehicle-treated) rats.

3.2.1. Ninety min

Ninety min after application, nerisopam led to widespread and dense Fos expression through the caudate-putamen (Fig. 1B, Fig. 2B, Fig. 3B). High density of Fos-like immunopositive cells was observed in the mediodorsal (Fig. 2B, Fig. 3B) (this area is probably equivalent to the 'body of the caudate nucleus' in primates and human) and in the most ventrolateral portion in the caudal sections of the caudate-putamen (Fig. 2B) (topographically equivalent to the 'tail of the caudate nucleus' in primates). Much lower density of Fos-like immunoreactivity was seen in the olfactory tubercle (Fig. 6) and in the nucleus accumbens which was mainly present in the shell portion (Fig. 7). Fos-like immunopositive cells were also found in the globus pallidus (Fig. 2B, Fig. 3B). Very few positive cells occurred in the compact zone of the substantia nigra (Fig. 4B) and none in the subthalamic or entopeduncular nuclei.

3.2.2. Five h

Five h after nerisopam administration still a small number of neurons established Fos-like immunoreactivity in the striatum, mainly in the mediodorsal part of it (Fig. 1C, Fig. 2C, Fig. 3C). Scattered Fos-positive cells were seen in the globus pallidus (Fig. 2C, Fig. 3C) and in the shell and the cone regions of the nucleus accumbens (not shown). In contrast to the early period, some of the neurons in the substantia nigra, both in the compact and reticular zones showed Fos-like immunopositivity (Fig. 4C). Neurons in the olfactory tubercle, the entopeduncular and subthalamic nuclei were virtually Fos-negative.

3.2.3. Chronic (13 days)

Although the animals were sacrificed 90 min after the last nerisopam application, the chronic (13 days) nerisopam treatment resulted in Fos-like immunoreactivity much less efficiently than the single dose. Fos-like immunopositive cells were visible mainly in the mediodorsal striatum (Fig. 1D, Fig. 2D, Fig. 3D) and in the lateral part of the substantia nigra (Fig. 4D). Cells in the compact and the reticular zones of the substantia nigra were virtually Fos-negative. Fos-like immunopositive cells were also found in the subthalamic nucleus and in the neighboring Forel's fields (Fig. 5B). In addition to the striato–pallido–nigral system, chronic nerisopam treatment induced Fos

Fig. 2. Fos-like immunoreactive neurons in the caudal portion (putamen) of the caudate-putamen and the globus pallidus. Coronal sections 0.7–0.8 mm caudal to the level of the bregma). (A) Control (vehicle-treated) rats. (B) High density of Fos-like immunopositive cells in the putamen, especially in the dorsomedial and the ventrolateral parts of the nucleus 90 min after nerisopam administration. Pallidal neurons establish intense Fos activity. (C) 5 h after nerisopam application. Low intensity and low density of Fos-like immunopositive cells in the putamen. No Fos-positive cells in the globus pallidus. (D) Chronic nerisopam treatment. Abbreviations: c, corpus callosum; cl, claustrum; cp, caudate-putamen; g, globus pallidus; v, lateral ventricle. Bar scale: 500 μm.

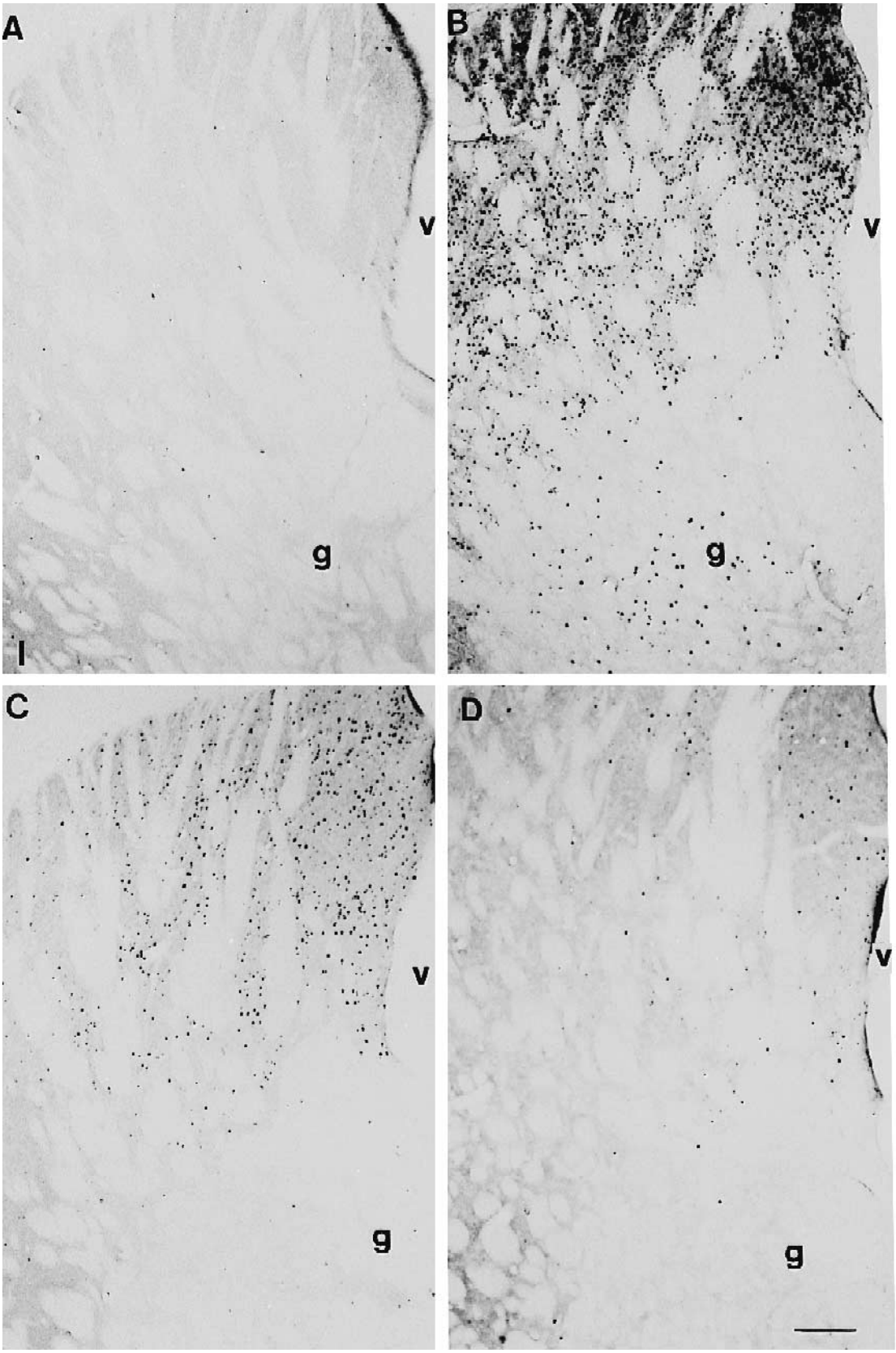

Fig. 3. Fos-like immunoreactive neurons in the globus pallidus and in the mediodorsal part of the caudate-putamen (body of the caudate nucleus). Coronal sections 0.6–0.8 mm caudal to the level of the bregma. (A) Control (vehicle-treated) rats. (B) Intense Fos-like immunoreactivity 90 min after nerisopam application. (C) Five h after nerisopam application. (D) Chronic nerisopam treatment. Abbreviations: g, globus pallidus; v, lateral ventricle. Bar scale: 200 μm.

expression in limbic brain areas like the hippocampus, the cingulate and piriform cortex, the lateral septal nucleus, as well as in the claustrum (Fig. 1D), the midline thalamic nuclei and brainstem catecholaminergic cell groups (not shown).

3.2.4. Unilateral transection of the striato–nigral connections

The unilateral transection of the striato–nigral connections failed to attenuate nerisopam-induced Fos activation in the striatum. Fos-like immunopositive cells appeared in both ipsi- and contralateral to the surgical transection (Fig. 8).

4. Discussion

Recent reports are indicating regional specificity in the effects of various antipsychotic drugs on c-*fos* expression in the basal ganglia. The typical and atypical antipsychotic drugs exert regionally distinct effects on striatal Fos expression (Miller, 1990; Robertson and Fibiger, 1992; Nguyen et al., 1992; Deutch et al., 1992; Merchant and Dorsa, 1993; Fink-Jensen and Kristensen, 1994; Wirtshafter and Asin, 1995; Semba et al., 1996). The typical antipsychotics, like haloperidol or fluphenazine, increase Fos expression in the dorsolateral and medial striatum (in both patch and matrix compartments) and in the core and shell portions of the accumbens nucleus. The atypical antipsychotics, like clozapine, sulpiride, risperidone or OPC-14597 have either no, or very moderate effects but only in the medial striatum, selectively in the patch compartment. Like the typical antipsychotics, the atypical ones increase Fos expression in the shell of the accumbens nucleus, but not in its core portion. In the present experiment, nerisopam induced a marked Fos expression in the striatum and had a moderate effect in the accumbens nucleus. The distribution pattern of the nerisopam-induced striatal Fos

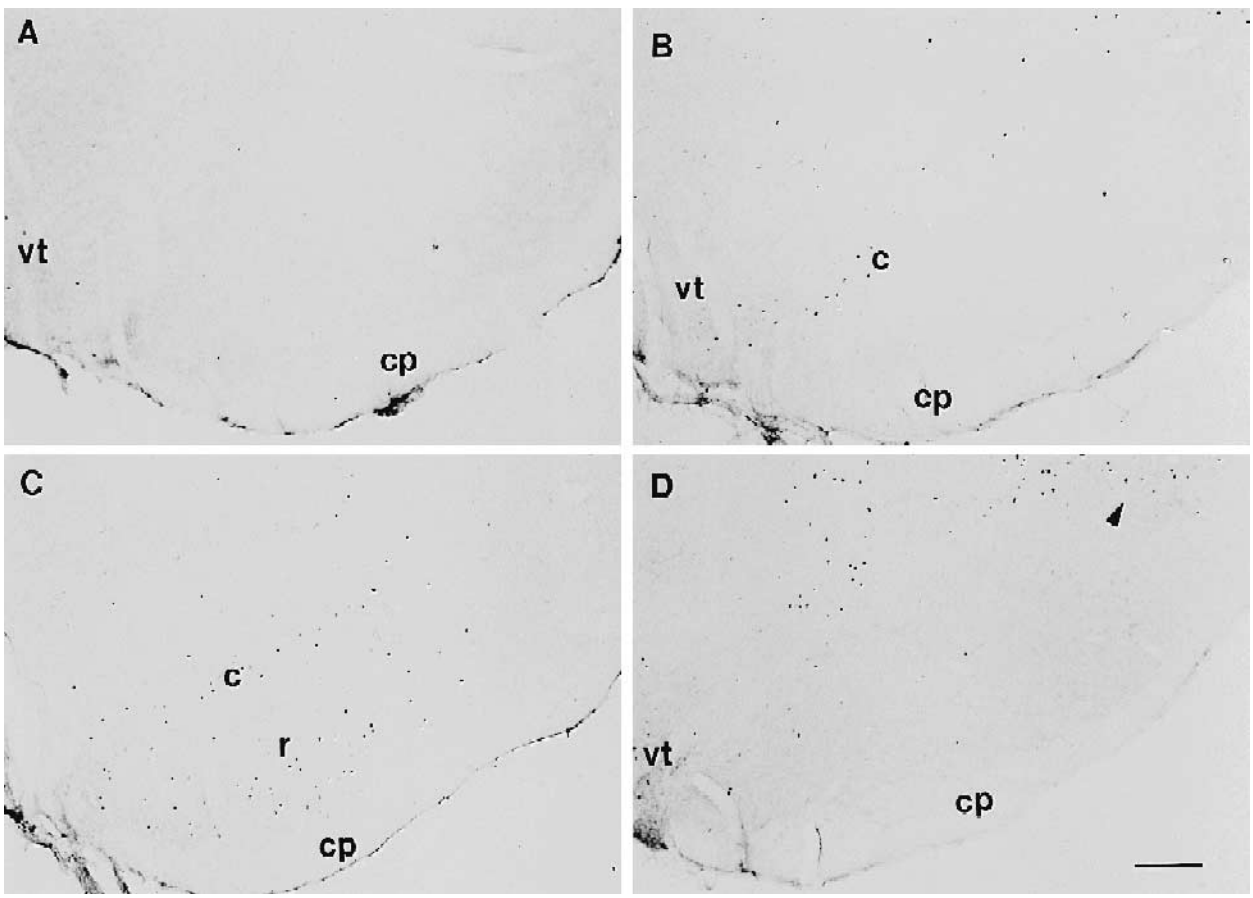

Fig. 4. Fos-like immunoreactive cells in the substantia nigra after nerisopam treatment. Coronal sections 5.5–5.7 mm caudal to the level of the bregma. (A) No Fos-like immunoreactive cells in the substantia nigra of the control animals. (B) Scattered Fos-positive cells in the zona compacta (c) of the substantia nigra 90 min after nerisopam treatment. (C) Fos-like immunoreactive cells both in the zona compacta and reticularis (r) of the substantia nigra 5 h after treatment. (D) No Fos-positive cells in the compact and reticular zones of the substantia nigra in rats with chronic (13 days) nerisopam treatment. The pars lateralis of the substantia nigra (arrowhead) contains few Fos-like immunopositive cells. Abbreviations: cp, cerebral peduncle; vt, ventral tegmental area. Bar scale: 200 μm.

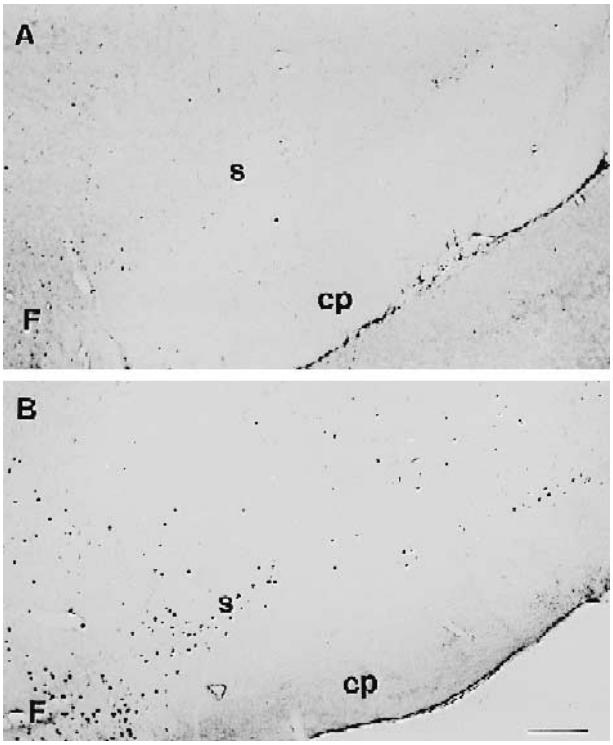

Fig. 5. Fos-like immunoreactive cells in the subthalamic nucleus (s) and in the fields of Forel (F). Coronal sections 3.7 mm caudal to the level of the bregma. (A) No Fos-positive cells in the subthalamic nucleus 90 min after nerisopam application. (B) Fos-positive cells after chronic (13 days) nerisopam treatment. Abbreviations: cp, cerebral peduncle. Bar scale: 200 μm.

expression, however, was distinct from that of haloperidol, the most marked changes occurred in the dorsomedial and the ventrolateral striatum. In the accumbens nucleus, nerisopam was effective like the atypical antipsychotics: it induced Fos expression in the shell but not in the core portion of the nucleus.

Nerisopam, like other homophthalazines, specifically bind to the basal ganglia, like caudate nucleus, putamen, nucleus accumbens, olfactory tubercle, globus pallidus, entopeduncular and subthalamic nuclei and the substantia nigra (Horváth et al., 1993, 1994). A single dose of i.p. administered nerisopam, however, induced marked c-*fos* expression in cells of the striatum and, less extensively in the nucleus accumbens and the pallidum but not in the projecting fields of these neurons, i.e. no Fos-like immunopositive cells were seen in the substantia nigra or in the subthalamic and entopeduncular nuclei. This observation, and the fact that surgical transection of the striato–nigral pathway prevents nerisopam bindings in the substantia nigra and the entopeduncular nucleus but not in the striatum (Palkovits et al., to be published) may indicate that the striatum and the pallidum but not the substantia nigra are the primary targets of nerisopam.

Striatal outputs can be divided into two major groups: striato–nigral neurons, which synthesize GABA, substance P, dynorphin and dopamine D_1 receptor, and striato–pallidal neurons containing GABA, enkephalin and dopamine D_2 receptor. It is generally believed that both striato–nigral and striato–pallidal neurons are controlled by the level of dopamine receptor stimulation. The striato–nigral neurons project direct to the substantia nigra while the striato–pallidal neurons constitute a part of the 'indirect' striato–nigral pathway via globus pallidus–subthalamic nucleus–substantia nigra. It has been proposed that dopamine acts on the dopamine D_1 receptors to stimulate striato–nigral neurons (mainly in the dorsomedial and lateral striatum), while it acts at the dopamine D_2 receptors to inhibit striato–pallidal neurons. Inhibition of striato–pallidal neurons may result in an activation of neurons in the subthalamic nucleus. Indeed, dopamine D_1 receptors influence c-*fos* expression in the subthalamic nucleus both in intact and striato–nigral lesioned rats (Ruskin and Marschall, 1995; Saji et al., 1995).

Neither dopamine D_1 receptor agonist (LaHoste et al., 1993), nor dopamine D_2 receptor agonist (Wirtshafter and Asin, 1994) induce c-*fos* expression in the striatum separately, but they have strong synergistic effects on striatal neurons (Paul et al., 1992; Wirtshafter and Asin, 1994). (Dopamine D_2 receptor antagonists, however, activate c-*fos* expression in the striatum (Rogue and Vincendon, 1992).) Apomorphine, a mixed dopamine D_1 and D_2 receptor agonist induces c-*fos* in the striato–nigral neurons (Cenci et al., 1992). In addition to dopamine receptor agonists and antagonists, muscarinic receptor (Bernard et al., 1993) and glutamate agonists (Berretta et al., 1992) can stimulate c-*fos* in the striatum, independently of each other. Nerisopam had similar but not identical effects to either dopamine receptor agonists, since it is very effective in Fos activation both on striatal (especially in the mediodorsal and ventrolateral part — like a dopamine D_1 receptor agonist, or a dopamine D_2 antagonist) and pallidal neurons (target site of dopamine D_2 receptor agonists (Paul et al., 1992; Wirtshafter and Asin, 1994)). Worth mentioning,

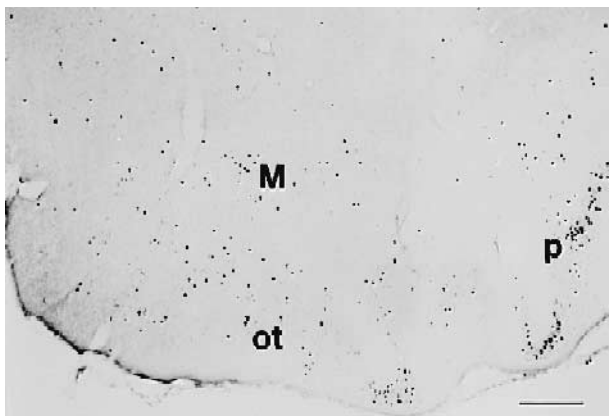

Fig. 6. Fos-like immunoreactivity in the olfactory tubercle (ot) and in the lateral preoptic area among the fibers of the medial forebrain bundle (M) after chronic (13 days) nerisopam treatment. High density of Fos-positive cells in the piriform cortex (p). Coronal section 0.2 mm rostral to the level of the bregma. Bar scale: 200 μm.

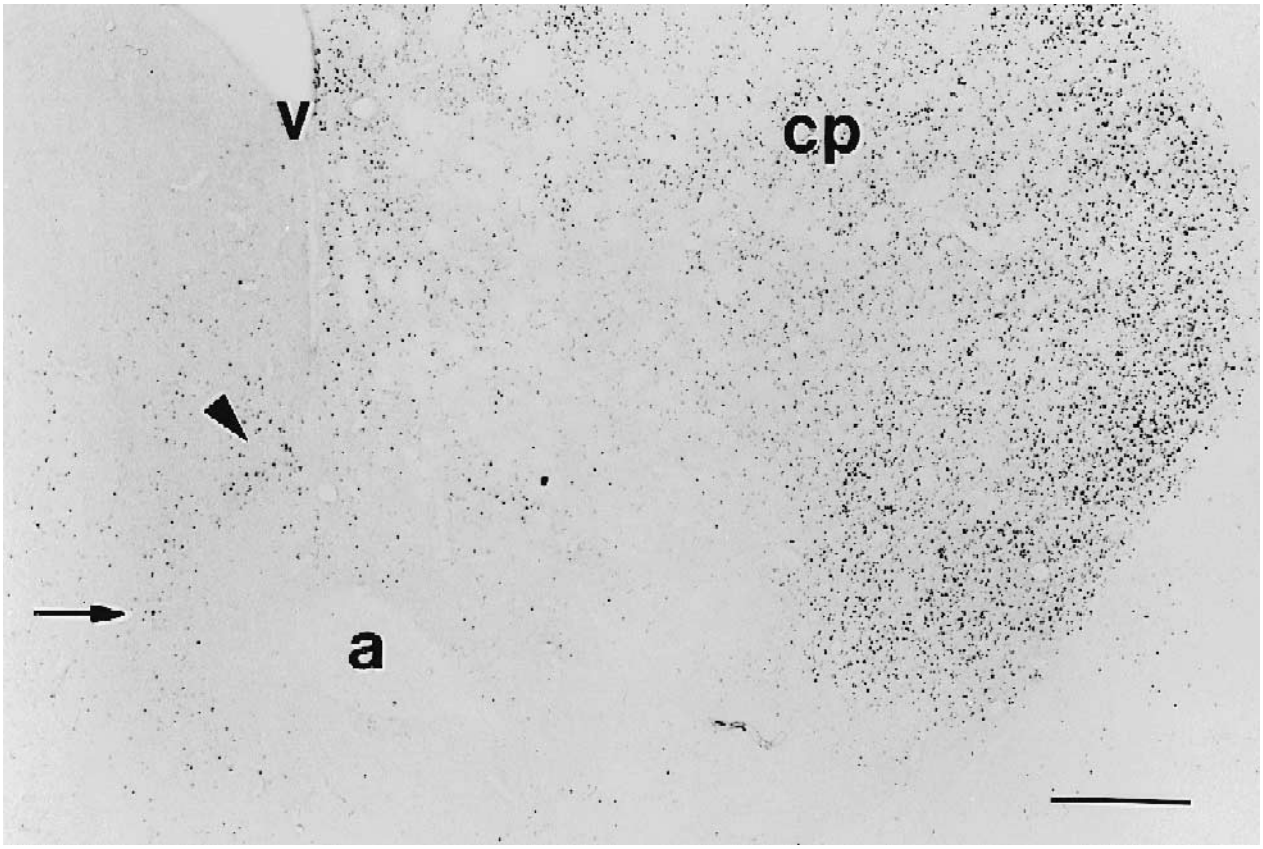

Fig. 7. Fos-like immunoreactive neurons in the cone (arrowhead) and the shell (arrow) portions of the nucleus accumbens 90 min after nerisopam application. Coronal section 0.9 mm rostral to the level of the bregma. Abbreviations: a, anterior commissure; cp, caudate-putamen; v, lateral ventricle. Bar scale: 400 μm.

that quinpirole, a dopamine D_2 receptor agonist, induces Fos expression in the globus pallidus but fails to exert any effect in the striatum (Robertson et al., 1992).

6-Hydroxydopamine-lesions of the nigrostriatal pathway, that reduce the ability of antipsychotics to induce Fos expression in the striatum and the accumbens nucleus (Robertson and Fibiger, 1992) failed to influence the nerisopam-induced Fos-like immunoreactivity in the striatum. Distinct to selective D_1 dopamine receptor agonists which induce c-*fos* expression in the 6-hydroxydopamine-denervated striatum ipsilateral but not contralateral to the injection (Robertson et al., 1989, 1990, 1992; Paul et al., 1992; Robertson and Fibiger, 1992; Asin and Wirtshafter, 1993), nerisopam induced Fos-like activation on both sides of the striatum in animals with striato–nigral transection.

Although nerisopam and haloperidol exerted quite similar actions on striatal and pallidal Fos expression, substantial differences were noted. A single injection of haloperidol has been shown to increase c-*fos* expression in the striatum (Dragunow et al., 1990; Miller, 1990; Robertson and Fibiger, 1992). Repeated daily, it continues to induce c-*fos* as efficiently as a single dose. Nerisopam produced a rapid and transient induction of Fos expression in the striatum, but only a slight elevation of Fos immunoreactivity was seen there after 13 days, daily application of nerisopam. Furthermore, the haloperidol-induced c-*fos* activation in the striatum can be abolished by lesioning of dopamine neurons with 6-hydroxydopamine (Ishibashi et al., 1996). Nerisopam may act on a different way since unilateral transection of the striato–nigral pathway, which eliminated tyrosine hydroxylase from the ipsilateral striatum failed to influence nerisopam-induced c-*fos* expression in striatal neurons. Haloperidol (Wirtshafter and Asin, 1995), but not nerisopam, induces c-*fos* expression in the substantia nigra and the entopeduncular nucleus. Like other atypical antipsychotic drugs (Deutch et al., 1992; Semba et al., 1996), nerisopam induced Fos activation in the nucleus accumbens. In contrast to haloperidol, Fos-like immunoreactive neurons occurred in the shell and cone, but not in the core region of the nucleus.

The substantia nigra is one of the principal output structures associated with the basal ganglia. It receives direct (striato–nigral) and indirect (striato–pallidal–subthalamic–nigral) inputs from the striatum. These two pathways have opposite effects on substantia nigra neurons. Activation of striato–nigral projecting neurons in the striatum results in an inhibition of cells in the pars reticulata of the substantia nigra. Activation of the striato–pallidal neurons results in disinhibition of the excitatory input from the subthalamic nucleus to the substantia nigra. The delayed (5

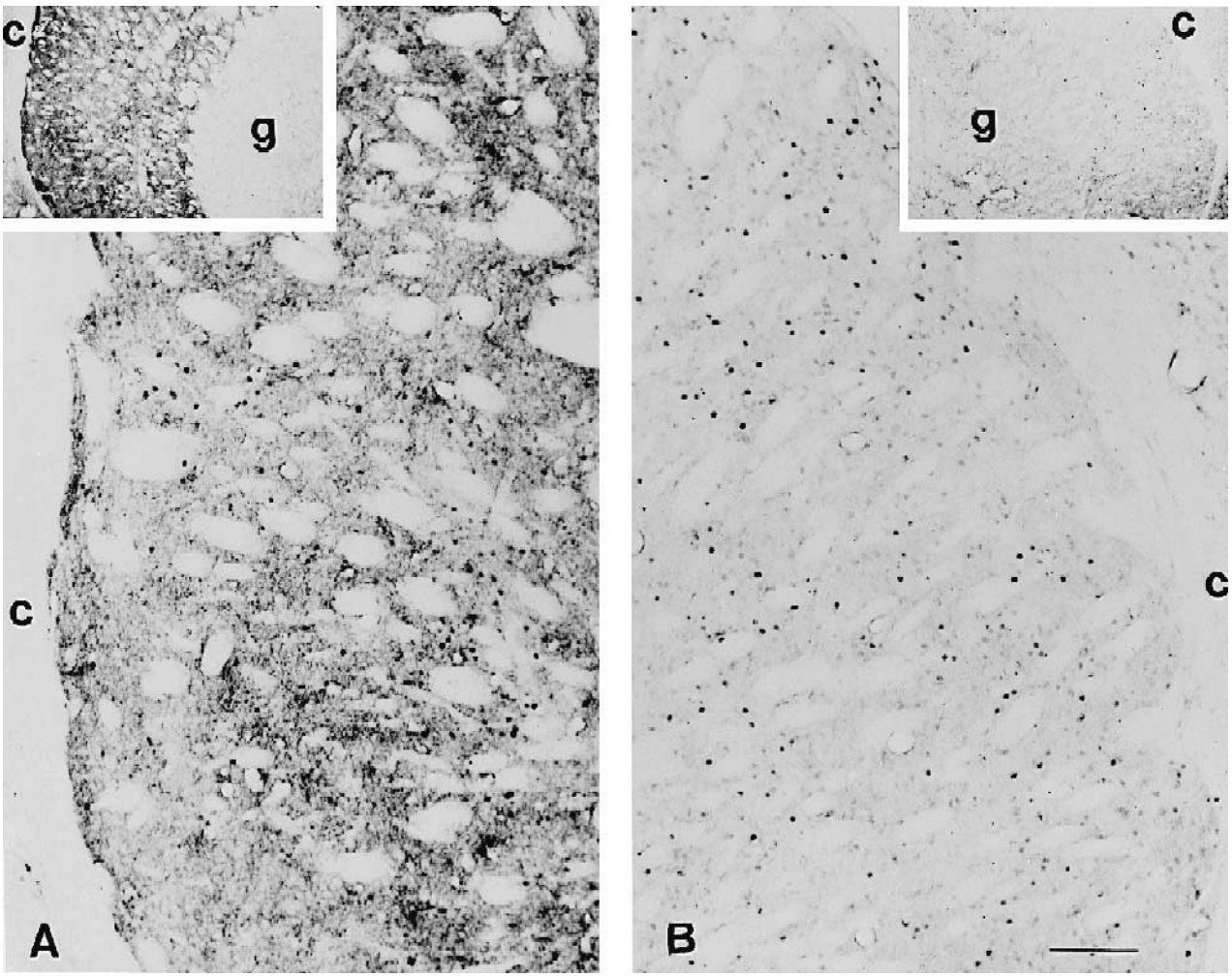

Fig. 8. Fos-like and tyrosine hydroxylase immunoreactivity in the caudate-putamen 90 min after nerisopam application in rats with unilateral surgical transection of the nigro–striatal pathway. Coronal sections 0.6–0.8 mm caudal to the level of the bregma. (A) Contralateral to the transection. (B) Ipsilateral to the transection. The comparable intensity of tyrosine hydroxylase-immunostaining in the putamen is shown by the inserts. The transection of the nigro–striatal pathway did not result in visible differences in the density of Fos-like immunopositive neurons in the putamen. Abbreviations: c, corpus callosum; g, globus pallidus. Bar scale: 100 μm.

h) effect of nerisopam on Fos expression in substantia nigra neurons may represent the second version: nerisopam, like other antipsychotics (Robertson et al., 1992) may activate the striato–pallidal neurons which inhibit neurons in the subthalamic neurons leading to overactivity of the substantia nigra.

Dopamine receptor antagonists induce Fos-like immunoreactivity in the entopeduncular nucleus (Wirtshafter and Asin, 1995). Nerisopam, with single or chronic applications, failed to elicit Fos activation in this nucleus.

There are several neuropeptides such as neurotensin, somatostatin and enkephalin which are transcriptionally regulated by c-*fos*. All of these neuropeptides are synthesized by striatal neurons (see Graybiel, 1990). Haloperidol induces c-*fos* expression in proenkephalin mRNA in striato–pallidal (Robertson et al., 1992) and neurotensin/neuromedin N mRNA in the dorsolateral striatal neurons (Merchant and Dorsa, 1993; Robertson et al., 1995). In order to clarify the chemical identity of cells in the striatum as well as in the other basal ganglia which expressed c-*fos* in response to nerisopam administration, combined c-*fos* and neuropeptide immuno-histochemical stainings are in progress.

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