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Effects of YM-43611, a Novel Dopamine D_2-Like Receptor Antagonist, on Immediate Early Gene Expression in the Rat Forebrain

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The pharmacological characteristics of two benzamides, YM-43611, a potent and selective dopamine D_3 and D_4 antagonist, and YM-09151-2 (nemonapride), were compared with two reference antipsychotic agents, haloperidol and clozapine, in terms of modification of c-fos *and related gene expression in the rat forebrain. After subcutaneous injection of YM-43611 (1 or 5 mg/kg), nemonapride (4 mg/kg), haloperidol (1 mg/kg), or clozapine (25 mg/kg), Fos immunocytochemistry was employed, and the distributions of Fos-like immunoreactive neurons were compared. As was the case for the two reference antipsychotics, the two benzamides enhanced c-Fos immunoreactivity in a number of forebrain regions. Specifically, like clozapine and nemonapride, YM-43611 significantly increased the number of immunoreactive cells in the nucleus accumbens shell and islands of Calleja. In contrast to clozapine and nemonapride, YM-43611 did not increase* c-fos *expression in the medial prefrontal cortex. Haloperidol and nemonapride elevated the number of positive cells in the striatum and nucleus accumbens core, whereas clozapine and YM-43611 did not. Clozapine increased the number of Fos-like immunoreactive cells in the lateral septal nucleus and the diagonal band nucleus, but YM-43611, nemonapride, and haloperidol did not. The present findings demonstrate that in comparison with three other drugs, YM-43611 has restricted effects on* c-fos *expression in the rat forebrain and is active primarily in the shell region of the nucleus accumbens and the islands of Calleja. The ability of YM-43611 to block D_3 and D_4 receptors may contribute to its unique actions on Fos induction.* ***[Neuropsychopharmacology 17:27–33, 1997]*** *© 1997 American College of Neuropsychopharmacology*

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The mechanism by which neuroleptics induce therapeutic effects remains elusive. Early receptor binding studies demonstrated a strong correlation between clinical potency and antagonism of the classical dopamine D_2 receptor (Creese et al. 1976; Seeman et al. 1976). Although recent pharmacological successes have implicated the potential role of several of the newly discovered dopamine receptors (D_3, D_4, D_5), nondopamine receptors, including serotonergic, adrenergic, and cholinergic receptors, are believed to subserve a specialized function in both the therapeutic action and side effect profiles of antipsychotic drugs (Deutch et al. 1991).

Immediate early genes (IEGs), such as *c-fos*, can be activated by a variety of physiological and pharmacological stimuli and mediate the synthesis of phosphoprotein Fos (Dragunow and Robertson 1987; Dragunow et al. 1987; Herschman 1989). Recently, it has been shown

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that haloperidol, a typical neuroleptic, and clozapine, an atypical antipsychotic, significantly increase neuronal *c-fos* and related gene expression in a number of forebrain regions (Dragunow et al. 1990; Deutch et al. 1992; Nguyen et al. 1992; Robertson and Fibiger 1992; MacGibbon et al. 1994; Fink-Jensen et al. 1995; Wan et al. 1995). These studies have indicated that typical and atypical antipsychotic drugs exert regionally distinct effects on neuronal IEG expression in the striatum, lateral septum, and islands of Calleja, although they exhibit quite similar actions on *c-fos* expression in the nucleus accumbens shell.

In addition to remoxipride, two benzamide derivatives have been introduced as selective antagonists for the dopamine D_2-like receptor. One is nemonapride, a pyrrolidiny-benzamide derivative (YM-09151-2) (Yamamoto et al. 1982), which is shown to have high affinity and selectivity for D_2-like receptors, such as the D_2, D_3, and D_4 receptors (Sokoloff et al. 1990; Van Tol et al. 1991; Seeman et al. 1993). Initial studies have indicated that nemonapride is effective against the positive symptoms and possibly against negative symptoms of schizophrenia (Mori et al. 1989; Satoh et al. 1996). The second is YM-43611, and this newly developed compound is a D_2-like receptor antagonist with negligible affinity for other neurotransmitter receptors (Hidaka et al. 1996; Ohmori et al. 1996). Nemonapride has a higher affinity for D_2 receptors than either D_3 or D_4 receptors, whereas YM-43611 exhibits a 5-fold greater selectivity for rat D_3 than D_2 receptors and 90-fold greater selectivity for rat D_4 than D_2 receptors; D_4 selectivity was an order of magnitude greater than that of clozapine (Hidaka et al. 1996).

To understand fully the mechanism of various actions of the neuroleptics, we investigated the acute effects of these benzamides on induction of IEG in the rat forebrain and compared them with those of haloperidol and clozapine. We particularly focused on regions that had been emphasized previously in relation to the drug action (e.g., striatum, lateral septum, nucleus accumbens, and islands of Calleja). The data on IEG induction in the cerebral cortex, diencephalon, and other regions will be dealt with in a different report.

MATERIALS AND METHODS

Drugs

Haloperidol (Dainippon, Osaka) and clozapine (Sandoz, Dorval, Québec) were dissolved in 40 ml of 20% acetic acid and were brought to final volume with distilled water (0.5 ml/kg). YM-43611, (S)-(+)-N-(1-benzyl-3-pyrrolidinyl)-5-chloro-4-cyclopropylcarbonylamino-2-methoxybenzamide (Yamanouchi), and nemonapride (emonapride; YM-09151-2), cis-N-(1-benzyl-2-methylpyrrolidin-3-yl)-5-chloro-2-methoxy-4-methyaminobenzamide (Yamanouchi, Tokyo) were dissolved in dilute hydrochloric acid (0.05 M).

Protocol

Male Wistar rats (body weight, 150–200 g) were group-housed (3 per cage) for 1 week before injections of haloperidol (1 mg/kg, $n = 6$), clozapine (25 mg/kg, $n = 6$), YM-43611 (0.1, 1.0, 5.0, 10.0 mg/kg, $n = 10$), nemonapride (0.5, 4.0 mg/kg, $n = 10$), or two vehicle solutions ($n = 12$). All of the injection solutions, including those for the controls, were matched on the basis of pH. Each animal received a single subcutaneous injection in the left thigh.

Fos Immunohistochemistry

Two hours after vehicle or drug injection, the rats were anesthetized with sodium pentobarbital (50 mg/kg IP) and were perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and placed in the same fixative for 24 hours. Thirty-micrometer-thick sections were cut from each brain with a vibratome. After a rinse in phosphate buffer saline, the sections were incubated with anti-c-Fos polyclonal antibody (Ab2: Oncogene Science, Uniondale, NY; diluted 1:2,000). This antibody has been shown to recognize N-terminal region of c-Fos and related proteins in the rat central nervous system (MacGibbon et al. 1994). The specificity of the immunoreactivity was examined by an absorption test using synthetic c-Fos peptide (N-terminal 16 amino acid residues; Cambridge Res. Biochem., Wilmington, DE, OP-11-3210). No immunoreactivity was observed in these control studies. The reaction products were detected using PK4001 kits (Vector, Burlingame, CA) and finally visualized by the diaminobenzidine-ammonium-nickel method. Some of the sections were counterstained with neutral red for anatomical verification, and the atlas of Paxinos and Watson (1986) was used as a reference.

Statistical Analysis

The number of c-Fos–like immunoreactive (FLI) cells were counted in a unit area (Figure 1). The differences between groups in the number of FLI cells were evaluated using one-way analysis of variance (ANOVA, Scheffe's F test) for each brain region (Table 1).

RESULTS

In comparison with vehicle-injected groups, low doses of YM-43611 (0.1 mg/kg) produced no significant changes in the distribution of FLI cells. Treatments with higher doses of YM-43611 (5, 10 mg/kg) resulted in similar distributions of FLI cells, as will be indicated.

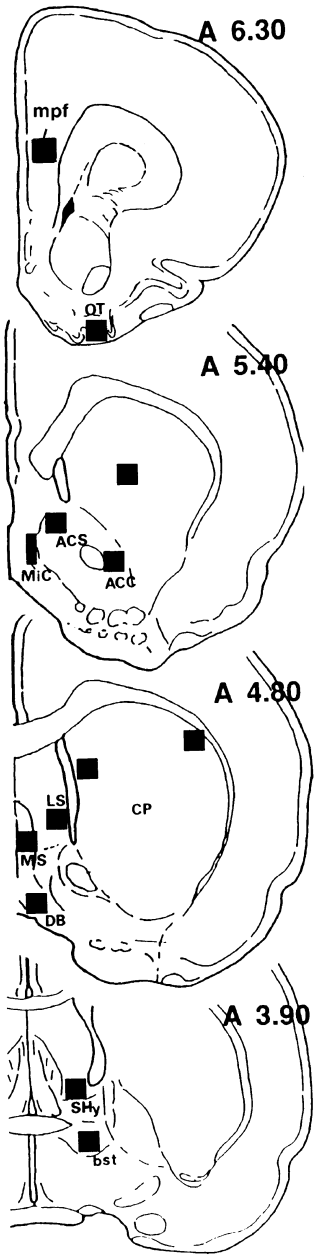

Figure 1. Areas to count Fos-positive neurons in the forebrain regions. Boxed regions indicate the 405-μm^2 sampled areas. Refer to the atlases of Paxinos and Watson (1986) and of Satoh et al. (1983) for anatomical nomenclature. ACS, nucleus accumbens shell; ACC, nucleus accumbens core; CP, caudate putamen; DB, diagonal band nucleus; MS, medial septal nucleus; MiC, major islands of Calleja; mpf, medial prefrontal cortex; LS, lateral septal nucleus; OT, olfactory tubercle; SHy, septohypothalamic nucleus; bst, bed nucleus of stria terminalis.

Septum and Other Medial Forebrain Regions

Significant increases in the number of FLI cells were observed in the lateral septal nucleus and diagonal band nucleus after treatment with clozapine (Figure 2A), but not with YM-43611, haloperidol, or nemonapride (Table 1). No significant increases in the number of positive cells were observed in the olfactory tubercle or the bed nucleus of stria terminalis in any of the drug-treated rats. Significant increases in the number of FLI cells were observed in the medial prefrontal cortex after treatment with clozapine and nemonapride, but not with YM-43611 or haloperidol (Table 1).

Nucleus Accumbens and Island of Calleja

In the nucleus accumbens shell, there were significant increases in the number of FLI cells after the injection of YM-43611 or either of the three antipsychotics (Figure 2B; Table 1). The distribution was similar for each drug, and the dorsal part of the shell contained relatively more FLI cells than the ventral part. On the other hand, significant increases in the number of positive cells were observed in the nucleus accumbens core after injection of haloperidol or nemonapride, but not after YM-43611 or clozapine (Table 1).

In the ventromedial basal forebrain, significant increases in the number of FLI cells were observed in the islands of Calleja of YM-43611–, clozapine-, or nemonapride-treated rats, particularly in the major island of Calleja (Figure 3A–3D; Table 1). The FLI cells were distributed heavily in the major island of Calleja. Haloperidol injection slightly enhanced the induction of positive cells in the islands of Calleja. In the counterstained sections of haloperidol-treated animals, the great majority of neurons in the major island of Calleja were not Fos positive.

Striatum

In the striatum of rats treated with either haloperidol or nemonapride, significant increases were observed in the number of FLI cells (Figure 3F and 3G; Table 1). YM-43611 injection resulted in a slight increase of FLI cells (Figure 3H). In contrast, clozapine had little effect on *c-fos* expression in the striatum.

DISCUSSION

In the present study, the effect of acute administration of the two benzamides, both selective dopamine D_2-like receptor antagonists, on IEG expression was investigated in the rat forebrain. The distribution of responding neurons, as reflected by the enhanced induction of Fos-like proteins, after a single injection of YM-43611 or nemonapride was compared with that seen in a typical neuroleptic, haloperidol, as well as an atypical antipsychotic, clozapine. The distributions of Fos-positive cells after haloperidol or clozapine treatment were similar, but not identical, to those previously reported (Dra-

Table 1. Effects of YM-43611 and Three Neuroleptics on the Number of Fos-Positive Cells within a 405-× 405-μm^2 Area of the Rat Forebrain Regions

Brain Regions	Vehicle	Haloperidol (1.0 mg/kg)	Clozapine (25 mg/kg)	Nemonapride (4.0 mg/kg)	YM-43611	
					1.0 mg/kg	5.0 mg/kg
Medial prefrontal cortex	8.8 ± 3.70	18.3 ± 8.64	22.0 ± 4.00*	35.2 ± 7.85*	11.2 ± 0.84	15.2 ± 8.44
Nucleus accumbens, shell	20.4 ± 4.39	58.5 ± 18.85*	43.8 ± 9.73*	39.0 ± 14.70*	41.8 ± 5.17*	53.6 ± 10.01*
Nucleus accumbens, core	1.0 ± 1.00	13.3 ± 7.09*	3.8 ± 5.28	16.0 ± 4.85*	1.4 ± 1.67	5.0 ± 5.24
Island of Calleja (major)	1.0 ± 2.24	82.8 ± 46.87	266.6 ± 116.09*	260.0 ± 53.50*	127.0 ± 46.98*	122.0 ± 54.61*
Diagonal band nucleus	1.4 ± 1.52	5.6 ± 2.37	14.4 ± 5.89*	8.0 ± 5.83	1.8 ± 1.64	4.0 ± 6.82
Lateral septum	23.4 ± 9.56	31.2 ± 10.94	78.2 ± 32.05*	29.2 ± 7.85	18.8 ± 8.26	43.2 ± 17.91
Medial septum	10.4 ± 4.93	17.4 ± 6.25	16.0 ± 6.46	5.8 ± 5.26	13.0 ± 6.63	11.4 ± 6.35
Septohypothalamic nucleus	21.8 ± 8.17	43.9 ± 18.04*	45.8 ± 18.79*	27.8 ± 9.18	12.0 ± 6.20	23.2 ± 10.94
Striatum (head)	0.2 ± 0.45	14.2 ± 7.99*	1.8 ± 6.30	18.2 ± 5.07*	2.2 ± 3.83	5.8 ± 2.17
Striatum (medial)	2.6 ± 2.07	14.7 ± 7.13*	3.8 ± 5.58	14.2 ± 2.49*	15.4 ± 4.93	11.0 ± 4.30
Striatum (lateral)	0.2 ± 0.45	27.9 ± 15.93*	0.6 ± 13.29	27.8 ± 6.72*	0.0 ± 0.00	5.2 ± 1.30
Bed nucleus of stria terminalis	2.4 ± 3.78	2.6 ± 0.75	3.2 ± 1.32	5.4 ± 3.21	0.6 ± 0.89	2.8 ± 3.03
Olfactory tubercle	1.8 ± 1.92	8.6 ± 3.89	5.2 ± 2.81	8.8 ± 3.83	6.2 ± 2.28	5.8 ± 3.03

Values are means ± SD and represent the average of five areas of different animals.
* $p < .05$, ANOVA with Scheffe's F test, versus controls.

gunow et al. 1990; Deutch et al., 1992; Robertson and Fibiger 1992; Nguyen et al. 1992).

The forebrain regions that were common to YM-43611, nemonapride, haloperidol, and clozapine with respect to induction of enhanced IEG expression were the nucleus accumbens and, with the exception of haloperidol, the islands of Calleja. However, it was only in the nucleus accumbens shell that all four drugs produced statistically significant increases in the number of FLI cells. The present finding that nemonapride is effective in such IEG induction extends earlier observations in rats (Robertson and Fibiger 1992; Deutch et al. 1992; MacGibbon et al. 1994) and monkeys (Ikemoto et al. 1995) and suggests that the nucleus accumbens shell may be a site of antipsychotic action.

A recent receptor binding autoradiographic study revealed one of the highest levels of D_3 binding in the nucleus accumbens (Booze and Wallace 1995), where the density of D_2 and D_3 receptor subtypes displayed a 1:1 ratio. Nemonapride has a high affinity and selectivity for D_2 like receptors (Terai et al. 1989); however, this drug is not selective for either D_3 or D_4 receptors over D_2 receptors (Hidaka et al. 1996). In contrast, YM-43611 exhibits moderate selectivity for D_3 receptors and high selectivity for D_4 receptors. Because both YM-43611 and nemonapride enhanced the induction of c-Fos–like proteins in the nucleus accumbens shell, it is conceivable that dopamine D_3 receptor antagonism, in addition to D_2 receptor antagonism, accounts for such an increase in IEG expression following drug administration. Previously, Robertson and Fibiger (1992) demonstrated that 6-OHDA lesions of the mesotelencephalic dopamine system block haloperidol- and clozapine-induced *c-fos* expression in the nucleus accumbens. Furthermore, Guo et al. (1995) have reported that pretreatment with 7OH-DPAT, a dopamine D_3 receptor agonist, reduces clozapine-induced *c-fos* expression in the nucleus accumbens shell. In situ hybridization histochemistry has revealed high levels of dopamine D_2 and D_3 receptor mRNA in the nucleus accumbens shell (Sokoloff et al. 1990; Mansour et al. 1990; Bouthenet et al. 1991; Diaz et al. 1995).

MacGibbon and colleagues (1994) demonstrated strong IEG (FRA, not c-Fos) induction in the islands of Calleja after clozapine treatment, but not after haloperidol injection. Guo et al. (1995) demonstrated that 7-OH-DPAT pretreatment significantly attenuated the clozapine-induced increase in the major island of Calleja. The present study confirmed these findings and further demonstrated that the two benzamides YM-43611 and nemonapride significantly increase the number of FLI

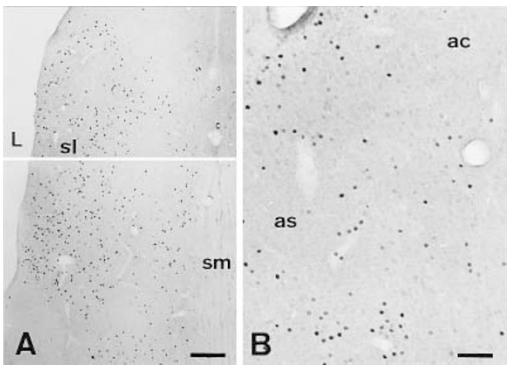

Figure 2. Fos-immunoreactivity in the septum and nucleus accumbens after injection of clozapine (25 mg/kg) (A) or YM-43611 (5 mg/kg) (B). L, lateral ventricle; ac, nucleus accumbens core; as, nucleus accumbens shell; sl, lateral septum; sm, medial septum. Bars: A, 200 μm; B, 50 μm.

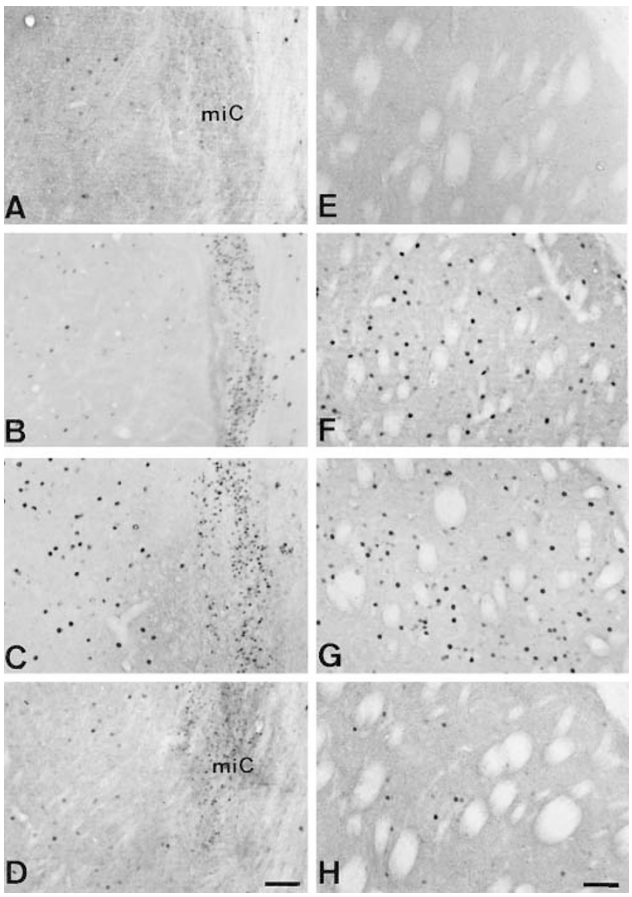

Figure 3. FLI neurons in (A–D) the major island of Calleja (miC) after injection of vehicle (A), clozapine (B), nemonapride (C), and YM-43611 (5 mg/kg) (D). Note the moderate intensity of Fos-like immunoreactivity in the major island of Calleja. Bars: 50 μm. (E–H) Fos-immunoreactivity in the lateral striatum following injection of vehicle (E), haloperidol (F), nemonapride (G), and YM-43611 (5 mg/kg) (H). Bars: 50 μm.

cells in the major island of Calleja. Haloperidol did not result in a significant increase of FLI cells in the major island of Calleja, although an increasing trend was observed. This dissimilarity between drugs likely originates from the difference in the pharmacological characteristics of these antipsychotic drugs. YM-43611, nemonapride, and clozapine, but not haloperidol, exhibit high affinity for dopamine D_3 and/or D_4 receptors (Sokoloff et al. 1990; Bouthenet et al. 1991; Van Tol et al. 1991). One of the highest levels of D_3 receptor binding was observed in the islands of Calleja (Hillefors-Berglund and Von-Euler 1994; Booze and Wallace 1995). In situ hybridization histochemical studies have shown that the islands of Calleja exhibit high dopamine D_3 receptor mRNA messages in the rat (Sokoloff et al. 1990; Bouthenet et al. 1991; Diaz et al. 1995).

A remarkable difference between the two benzamides in terms of IEG induction occurred in the striatum. Like haloperidol, nemonapride markedly enhanced the number of FLI neurons in the striatum, but YM-43611 at the present doses did not. This differential induction could be brought about by different profiles in D_2 receptor blockade, as the striatum has high concentrations of D_2 receptors (Booze and Wallace 1995). In contrast to nemonapride, YM-43611 is a D_2-like receptor antagonist with a 5-fold lower affinity for rat D_2 than D_3 receptors; such a pharmacological characteristic may account for failure to enhance intense striatal IEG expression. Similarly, an increase in Fos immunoreactivity was observed in the nucleus accumbens core following nemonapride and haloperidol administration, but not after YM-43611 or clozapine treatment.

In the septum, haloperidol and clozapine produced significant increases of FLI-positive cells in the septohypothalamic nucleus, but clozapine alone induced a significant increase of FLI cells in the lateral septum. Previous studies (Robertson and Fibiger 1992; MacGibbon et al. 1994) have shown that the number of FLI neurons in the lateral septal nucleus (ventral part) are increased by haloperidol as well as clozapine (higher c-Fos induction by clozapine than haloperidol), although data on the adjacent septohypothalamic nucleus were not presented. The reason for the discrepancy between the present and previous studies is not clear. It may arise from a difference in the antibody used. Moreover, as the distribution pattern of FLI cells was uneven in the lateral septal area (including the septohypothalamic nucleus), the discrepancy might merely be a sampling effect (different region of interest).

The present results are in accord with previous observations (Robertson and Fibiger 1992; MacGibbon et al. 1994) that clozapine enhances IEG expression in the ventral septum. Unlike clozapine, the two benzamides failed to affect IEG expression in the lateral septal area. The reason for this difference is uncertain. Although clozapine has antagonist effects on D_2-like receptors, it also has effects on other receptors, such as serotonergic (Canton et al. 1990), cholinergic, and adrenergic receptors (Tamminga and Gerlach 1987; Baldessarini et al. 1992). These antagonist properties of clozapine on nondopaminergic receptors might result in enhancing IEG expression in the lateral septum.

In the medial prefrontal cortex, clozapine and nemonapride produced significant increases in the number of FLI cells, whereas YM-43611 and haloperidol did not. Guo et al. (1995) demonstrated that pretreatment with 7-OH-DPAT, a D_3 agonist, did not significantly decrease clozapine-induced IEG expression in the medial prefrontal cortex and raised the possibility that the primary mechanism by which clozapine increases IEG expression in the prefrontal cortex might be due to antagonist actions at D_4 receptors. However, a recent pharmacological study argued against simple D_3 or D_4 antagonism as the cause of such changes in IEG expres-

sion in the medial prefrontal cortex (Deutch and Duman 1996), as many D_2-like receptor antagonists do not change *c-fos* expression in this region. The present study, taken together with previous reports, suggest that simple D_2-like receptor antagonism is not sufficient to induce IEG expression in the medial prefrontal cortex.

CONCLUSION

YM-43611 treatment enhanced c-Fos immunoreactivity in selective regions of the rat forebrain. As YM-43611 produced an increase in the number of FLI cells in the nucleus accumbens shell and islands of Calleja, as some other antipsychotics did, it is hypothesized that this benzamide has potential as an antipsychotic drug. YM-43611 shows a moderate selectivity for the dopamine D_3 receptor (K_i 35.5 nM) and a high selectivity for D_4 receptor (K_i 1.85 nM) (Hidaka et al. 1996). Although antagonist actions at D_3 receptors may account for the ability of YM-43611 to increase FLI in the nucleus accumbens shell and the islands of Calleja, the extent to which D_4 blockade contributed to the present IEG results is not clear. D_4 receptor mRNA is localized more abundantly in the cerebral cortex, hypothalamus, and amygdala than in the regions studied here (O'Malley et al. 1992). The present study points to the need for future studies using highly selective D_4 receptor antagonists.

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