

Effects of Aripiprazole and Haloperidol on Fos-like Immunoreactivity in the Prefrontal Cortex and Amygdala

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Objective: Aripiprazole, a dopamine system stabilizer, shows efficacy against both negative symptoms and positive symptoms in patients with schizophrenia. The aim of this study was to investigate the effects of aripiprazole and haloperidol on c-FOS expression in rat brain.

Methods: Aripiprazole (1, 10 and 30 mg/kg, i.p.) and haloperidol (0.1 and 1 mg/kg, i.p.) were administered to adult Male Sprague-Dawley rats. After 2 h of drug or vehicle administration, the rats were killed and their brains were removed and perfused with fixative, then cut into 40 μ m slices on a freezing microtome. Brain regions of interest were the medial prefrontal cortex (mPFC), the nucleus accumbens core and shell (NAC-C and NAC-S), the hippocampus (CA1, CA3 and DG), the central amygdala (Ce), the basolateral amygdala (BL) and the temporal cortex (Tc). Immunohistochemistry was performed to label cell bodies containing c-FOS.

Results: The administration of aripiprazole at all doses (1, 10 or 30 mg/kg) resulted in greater Fos-like immunoreactivity (FLI) in the investigated brain areas, as compared to the vehicle. Comparable increases in FLI were demonstrated in the NAC-C and NAC-S in response to both aripiprazole and haloperidol treatment. The administration of haloperidol (0.1 or 1 mg/kg) also resulted in greater FLI in the investigated brain areas, except the mPFC, where no changes were observed. In the Ce and BL, a significant increase in Fos-positive neurons was observed only with 0.1 mg/kg of haloperidol.

Conclusion: Both aripiprazole and haloperidol increased FLI in limbic areas, which are considered important targets of anti-psychotic drugs. The differential action of aripiprazole on FLI in the amygdala and mPFC as compared to haloperidol may be a good way to differentiate atypical from typical antipsychotics.

KEY WORDS: Aripiprazole; c-FOS; Haloperidol.

INTRODUCTION

The proto-oncogene *c-fos* has received special attention within the neurophysiological community as it may serve as a useful marker of brain regions activated following various physiological stimuli. Because the expression of Fos, the protein product of *c-fos*, is correlated with neuronal activation, Fos immunohistochemistry (IHC) has become a useful tool to map the functional pathways of the central nervous system. In particular, pharmacological studies suggest that Fos IHC can be used to identify the

potential neuroanatomical sites of drug action. Using this methodology, it has been shown that typical and atypical antipsychotics induce the same pattern of Fos-like immunoreactivity (FLI) in the nucleus accumbens (NAC) and lateral septum. In contrast, differential patterns of FLI arise in the striatum and prefrontal cortex (PFC) in response to antipsychotics that are predictive of whether a drug causes extrapyramidal side effects and is effective in treating the negative symptoms of schizophrenia. Robertson *et al.*¹⁾ claimed that typical and atypical antipsychotics could be distinguished on the basis of an index obtained by subtracting the extent of FLI induction in the dorso-lateral striatum from FLI in the NAC. If the index is negative, the drug is a typical antipsychotic, and if positive, an atypical antipsychotic. Nevertheless, in most previous studies the neuroleptics examined were limited to haloperidol or clozapine and the brain regions examined were mainly stri-

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tum or NAC. We have a special interest in the amygdala (Amyg), temporal cortex (Tc), and hippocampus (HIP) regions and therefore included these regions in the present study. The Amyg plays a central role in neuronal signaling of fear memory, which is closely implicated in the pathogenesis of schizophrenic symptoms. The Tc has recently been proposed as a common site of action for antipsychotics based on the finding that clozapine resembles other antipsychotics in its ability to cause high levels of D₂ receptor occupancy in the temporal cortex.^{2,3} The HIP abnormalities have been reported in the postmortem studies in schizophrenia.⁴ Chung *et al.*⁵ suggested that clozapine's superior action on cognition compared to haloperidol may be related to its greater action on the release of dopamine in the HIP.

Aripiprazole is a recently introduced, second-generation antipsychotic that has a receptor-binding profile that is distinct from those of other second-generation antipsychotics. This drug is a partial agonist at D₂ and 5-hydroxytryptamine (5-HT)_{1A} receptors and is also an antagonist at 5-HT_{2A} receptors. Only a few studies have been conducted with regard to the effects of aripiprazole on c-FOS expression: FOS expression for aripiprazole in the accumbens shell became observable only at occupancies exceeding 80% D₂ receptor occupancy⁶ and an increase in expression of the *c-fos* mRNA in the striatum was not demonstrated by aripiprazole.⁷ However, action of aripiprazole in the Amyg, Tc and HIP has never been investigated using Fos IHC. The objectives of the present study were to evaluate the induction of c-Fos by measuring FLI in areas of the rat brain, including the Amyg, Tc and HIP after acute administration of aripiprazole and haloperidol.

METHODS

Animals

Adult male Sprague-Dawley rats (Orient Bio Inc, Korea), weighing 200-250 g, were used. They were housed in groups of 3-4 in a temperature-controlled room under light/dark cycle (lights on from 7:00-19:00) with food and water ad lib. All possible efforts were made to minimize animal suffering and the number of animals used, in accordance with the Guidelines for Animal Experiments of Chonbuk National University Medical School.

Drugs

Haloperidol was purchased from Sigma (USA) and ari-

piprazole was generously provided by the Otsuka Pharmaceutical Company (Japan). Haloperidol and aripiprazole were dissolved in 0.1 M tartaric acid and 45% 2-hydroxypropyl- β -cyclodextrin (HBC) (Sigma, USA), respectively. All solutions were adjusted to a final pH of 5-6 with NaOH. Thus, the study was comprised of the following four groups: aripiprazole, haloperidol and two controls (45% HBC and 0.1 M TTA). Drug groups received one of the following five treatments: aripiprazole (1, 10 or 30 mg/kg) or haloperidol (0.1 or 1 mg/kg). All solutions were freshly prepared and administered intraperitoneally at a volume of 1 ml/kg. The doses were chosen on the basis of therapeutic equivalency for haloperidol and previous reports for aripiprazole.^{6,8} To minimize the influence of stress on the induction of FLI, rats were habituated to the manipulation that preceded the injection for 4 days before the experiment. Rats were also kept in a familiar cage in the animal room during the experiment. Two hours after injection, all of the animals were deeply anesthetized with a ketamine/xylazine mixture (4 : 1) and were perfused first with 200 ml of calcium-free Tyrode's solution and then with 200 ml ice-cold 4% paraformaldehyde in 0.1 M TPBS through the left cardiac ventricle to remove circulating blood. Each brain was removed immediately after perfusion, placed in fresh fixative for at least 4 h and then transferred to sucrose solutions (20% for 12 h followed by 30% for 24 h) in 0.1 M TPBS. The brains were embedded with OCT in liquid nitrogen and kept at -80°C until further use.

Immunohistochemistry

Coronal, 40- μ m-thick sections were cut using a cryostat microtome. Regions of interest in the brain were the prefrontal cortex (PFC), nucleus accumbens core and shell (NAC-C and NAC-S), hippocampus (CA1, CA3 and DG), central amygdala (Ce), basolateral amygdala (BL), and temporal cortex (TC). Cryoprotected, fixed sections were washed three times in TPBS and then bathed for 10 min in 0.3% H₂O₂ to block endogenous peroxidase. Sections were blocked in 3% normal goat serum and 0.3% triton X-100 in TPBS for 1 h, and then washed and incubated overnight at 4°C with polyclonal rabbit anti-*c-fos* antibody (1 : 10,000) (Calbiochem, USA). The sections were washed three times with TPBS and then incubated for 1 h. with rat adsorbed biotinylated anti-rabbit IgG (Vector, USA) diluted 1 : 200 in a solution containing 3% normal goat serum and 0.3% triton X-100 in TPBS. Finally, the sections were washed, incubated with ABC reagents (Vector, USA) for 1 h, and FLI visualized using

Table 1. Number of Fos-like positive neurons after acute administration of aripiprazole in rat brain

Brain regions	Vehicle (n=8)	ARI 1.0 mg/kg (n=8)	ARI 10.0 mg/kg (n=8)	ARI 30.0 mg/kg (n=8)	p-value	ANOVA followed by Scheffe test					
						Vehicle vs. ARI 1.0	Vehicle vs. ARI 10.0	Vehicle vs. ARI 30.0	ARI 1.0 vs. ARI 10.0	ARI 1.0 vs. ARI 30.0	ARI 10.0 vs. ARI 30.0
mPFC	33.23±3.23	50.44±6.77	64.41±3.69	58.78±8.02	0.004	NS	*	*	NS	NS	NS
NAC-C	6.95±1.76	25.81±5.80	40.56±6.32	43.62±7.82	<0.001	NS	†	†	NS	NS	NS
NAC-S	8.27±1.15	22.38±5.27	38.48±4.99	46.12±7.31	<0.001	NS	*	†	NS	*	NS
CA1	10.16±0.97	23.61±4.28	18.14±1.55	34.22±7.10	0.004	NS	NS	*	NS	NS	NS
CA3	7.56±1.59	11.62±1.21	19.17±1.62	37.78±7.93	<0.001	NS	NS	†	NS	*	*
DG	18.98±2.54	33.49±4.15	38.33±3.45	52.80±5.87	<0.001	*	*	†	NS	*	NS
Ce	15.44±1.81	19.89±1.98	30.72±2.93	42.84±7.56	<0.001	NS	*	*	NS	*	NS
BL	11.09±1.47	19.38±1.41	21.01±1.60	25.56±2.04	<0.001	*	*	†	NS	NS	NS
Tc	24.42±3.16	38.09±6.83	32.25±2.00	89.40±7.33	<0.001	NS	NS	†	NS	†	†

Vehicle, 45% 2-hydroxypropyl- β -cyclodextrin; ARI, aripiprazole; mPFC, medial prefrontal cortex; NAC-C, nucleus accumbens core; NAC-S, nucleus accumbens shell; DG, dentate gyrus; Ce, central amygdala; BL, basolateral amygdala; Tc, temporal cortex; NS, non-significant. * $p < 0.05$; † $p < 0.001$.

3,3-diaminobenzidine (Sigma, USA) and 0.2% nickel chloride intensification. The sections were mounted on gelatin-subbed slides and the slides were dried, dehydrated in ethanol (70-100% gradually), cleared in xylene, and coverslipped.

Quantification

FLI was analyzed using MetaMorph[®] image analysis software (Universal Imaging, West Chester, PA, USA) by a single, treatment-blind observer. Sections were viewed at 100 \times magnification and FLI staining was assessed within rectangular grids of defined size: 300 \times 500 μ m² (NAC-C and NAC-S), 400 \times 400 μ m² (Ce and BL), 500 \times 500 μ m² (mPFC and Tc), 0.2 mm² (CA1 and CA3), 0.6 mm² (DG). A grid of the predetermined size was placed in each brain region at a fixed position according to the locations of anatomical landmarks. The software calculated average gray-scale levels of background staining for each section. Then, FLI-positive nuclei were automatically counted within the grid if their pixels were >30 gray-scale levels darker than the average gray-scale level of each section. This threshold difference was kept constant for all of the digitally captured images. Manual correction was made to the automated counting for each section as necessary. Only darkly labeled, oval-shaped nuclei that matched the above criteria were counted. Counts obtained from the left and right hemispheres in three adjacent sections through an area of interest were averaged to generate one measurement of Fos-positive nuclei per region. Alternate sections, incubated in the absence of primary antibody as an immunohistochemical control, showed no immunostaining.

Data Analysis

Data are presented as mean \pm SEM for each treatment group. For statistical analysis, a one-way analysis of variance (ANOVA) was performed on the number of FLI-positive nuclei within a specified brain region to compare the aripiprazole and haloperidol treatment groups. Post-hoc individual comparisons were made using Scheffe's test. Differences were considered statistically significant at the $p < 5\%$ level.

RESULTS

Effects of Acute Aripiprazole Administration on Fos Immunoreactivity

The mean number of Fos-positive neurons in each brain region after acute aripiprazole treatment is shown in Table 1. Representative photomicrographs of FLI in select brain regions are shown in Fig. 1. Rats treated with aripiprazole at all doses showed significantly more Fos-expressing neurons in all brain regions examined compared to the vehicle-treated controls. In the NAC-S, CA3, DG, Ce, and Tc, dose-dependent increases in the number of Fos-positive neurons were observed. In the DG and BL, significant FLI was observed even at a small dose, 1 mg/kg of aripiprazole. However, in the CA1, CA3, and Tc, only 30 mg/kg of aripiprazole produced significant increases of FLI.

Effects of Acute Haloperidol Administration on Fos Immunoreactivity

The effects of acute haloperidol treatment on FLI are shown in Table 2 and Fig. 2. The patterns of FLI induced by acute haloperidol and aripiprazole administration were distinctly different. Acute administration of haloperidol at

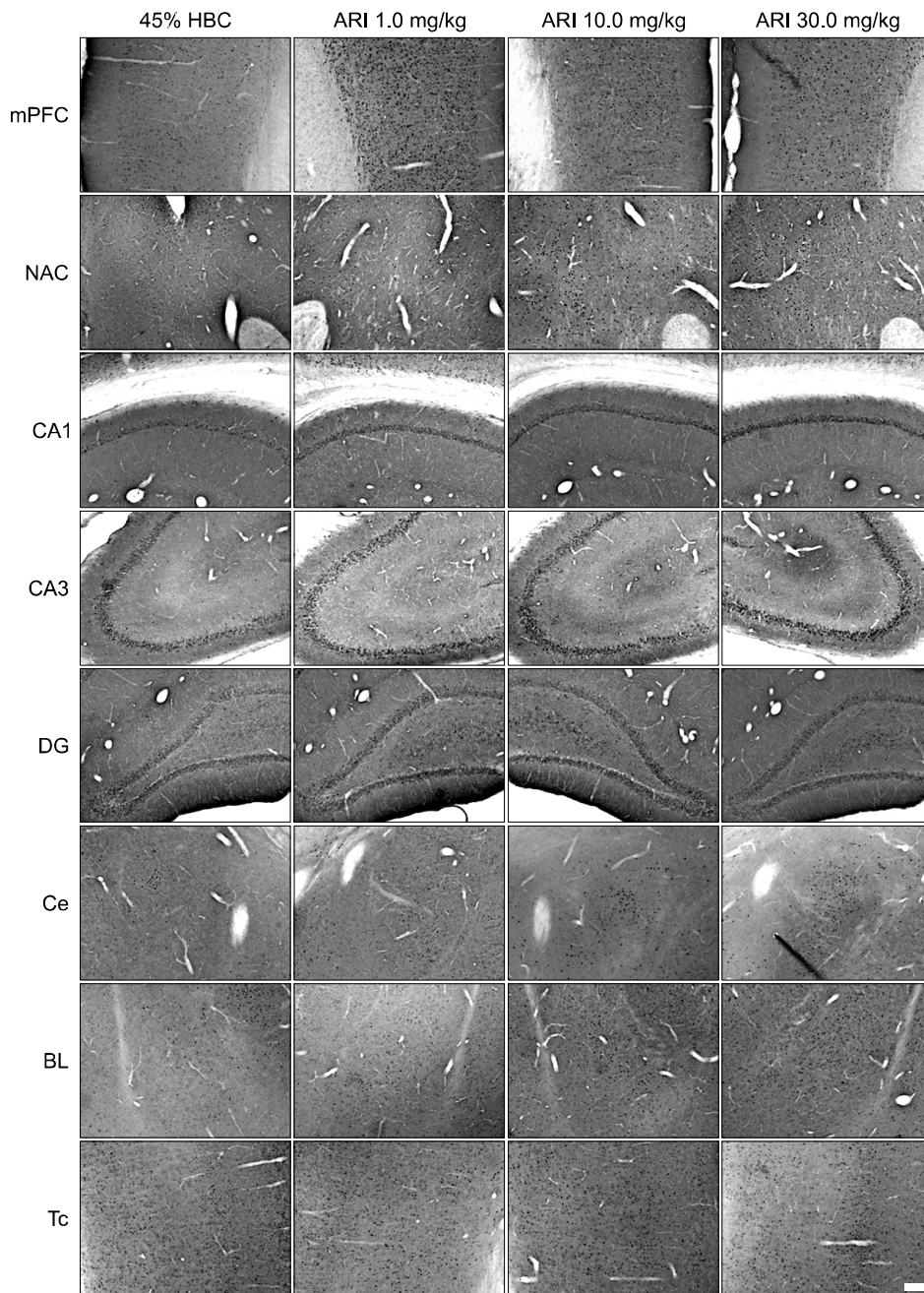


Fig. 1. Representative photomicrographs illustrating the number of Fos-positive neurons in the rat brain after single exposure to antipsychotic agents. HBC, 2-hydroxypropyl- β -cyclodextrin; ARI, aripiprazole-treated; mPFC, medial prefrontal cortex; NAC, nucleus accumbens; DG, dentate gyrus; Ce, central amygdala; BL, basolateral amygdala; Tc, temporal cortex. N=8 rats/group. Scale bar: 100 μ m.

0.1 or 1 mg/kg induced a significant increase in Fos-positive neurons compared with vehicle-treated controls in most brain areas examined except the mPFC, where no changes were observed with administration of either 0.1 or 1 mg/kg of haloperidol. The number of Fos-positive neurons was higher after administration of 1.0 mg/kg than after 0.1 mg/kg of haloperidol in the NAC-S, CA3, DG, and Tc. In the Ce and BL, a significant increase in Fos-positive neurons over vehicle-treated controls was observed only after administration of 0.1 mg/kg of

haloperidol.

DISCUSSION

Using an IHC technique, the results of the present study show that acute administration of aripiprazole and haloperidol both increased expression of c-Fos in a wide range of brain areas including the Amyg and Tc.

Both aripiprazole and haloperidol treatments caused similar increases in FLI in the NAC-C and the NAC-S.

Table 2. Number of Fos-like positive neurons after acute administration of haloperidol in rat brain

Brain regions	Vehicle (n=8)	HAL 0.1 mg/kg (n=8)	HAL 1.0 mg/kg (n=8)	p-value	ANOVA followed by Sheffe test		
					Vehicle vs. HAL 0.1	Vehicle vs. HAL 1.0	HAL 0.1 vs. HAL 1.0
mPFC	34.97±5.00	46.76±1.95	38.53±2.89	0.074	NS	NS	NS
NAC-C	6.68±0.73	24.66±1.83	28.15±1.86	0	†	†	NS
NAC-S	10.08±1.59	29.83±2.72	46.75±4.36	0	†	†	*
CA1	18.52±1.14	39.44±5.28	44.88±7.40	0.005	*	*	NS
CA3	5.06±0.62	14.52±1.20	40.31±3.33	0	*	†	†
DG	14.74±1.59	20.28±1.22	61.09±5.32	0	NS	†	†
Ce	15.44±2.02	43.38±2.23	15.44±1.08	0	†	NS	†
BL	16.66±1.14	33.44±1.99	18.09±2.13	0	†	NS	†
Tc	28.00±2.14	49.59±4.79	99.75±13.52	0	NS	†	*

Vehicle, 0.1 M tartaric acid; HAL, haloperidol; mPFC, medial prefrontal cortex; NAC-C, nucleus accumbens core NAC-S, nucleus accumbens shell DG, dentate gyrus; Ce, central amygdala; BL, basolateral amygdala; Tc, temporal cortex; NS, non-significant. * $p < 0.05$; † $p < 0.001$.

These results are in contrast with previous reports^{6,9)} that haloperidol induced comparable c-Fos expression in both the core and the shell of the NAC, whereas atypical antipsychotics such as clozapine, sulpiride, and aripiprazole produced greater c-Fos expression in the NAC-S than in the NAC-C. Evidence suggests that dopaminergic innervation of the NAC-C is associated with the nigrostriatal system while that of the NAC-S is associated with the mesolimbic system.¹⁰⁾ Therefore, it has been suggested that the preferential effect of atypical antipsychotics on the NAC-S over the NAC-C may explain the lower propensity of atypical antipsychotics to cause extrapyramidal motor side effects. As aripiprazole causes few extrapyramidal motor side effects, we expected a preferential effect of aripiprazole on the NAC-S, which was not the case. The explanation for this is not clear at the present. One difference is that Semba *et al.*¹¹⁾ measured c-fos mRNA expression instead of the protein product, Fos, that was measured in our study. To investigate the mechanism by which aripiprazole results in few extrapyramidal motor side effects further, measurement of c-fos expression in the dorsolateral striatum should be included in future studies.

We obtained different results in the mPFC with aripiprazole and haloperidol treatments. Aripiprazole treatment induced a significant increase in FLI in the mPFC, but haloperidol treatment did not. Our finding that there was no effect of haloperidol on FLI in the mPFC is in agreement with previous studies.^{1,12)} To the best of our knowledge, this is the first report to demonstrate a significant increase in FLI in the mPFC resulting from aripiprazole. Other findings regarding the action of aripiprazole in the PFC come from microdialysis studies,^{13,14)} in which a low dose of 0.3 mg/kg aripiprazole produced a

significant increase in dialysate dopamine levels in the mPFC. Together, these findings lend supporting evidence to the idea that atypical antipsychotics alleviate the negative symptoms of schizophrenia by activation of the mPFC. However, it should be noted that the results of some previous studies oppose this idea. In one study, continuous arterial spin-labeling magnetic resonance imaging did not measure a change in blood perfusion to the mPFC in response to aripiprazole treatment,⁸⁾ and in another study extracellular dopamine levels in mPFC dialysates did not change significantly after aripiprazole treatment.^{11,15)}

The major finding of the present study is that both aripiprazole and haloperidol significantly increased FLI in the HIP, Tc, and Amyg. In the HIP and Tc, high doses of aripiprazole (30 mg/kg) and haloperidol (1 mg/kg) produced the greatest increase in FLI. There have been conflicting results with regard to the effects of antipsychotics in the Tc: single injections of haloperidol and metoclopramide did not alter Fos expression in the inferior Tc;¹⁶⁾ a 14-day treatment with clozapine or haloperidol produced increased or decreased levels of D₂ mRNA, respectively;¹⁷⁾ and, a 6-month treatment with various antipsychotics increased the level of mRNA encoding the D₂ dopamine receptor in the Tc.¹⁸⁾ Nevertheless, the new proposal that the Tc is a common site of action for antipsychotics is of great interest. Many neuroimaging studies suggest that the Tc is a key area associated with the pathogenesis of auditory hallucination, and 25-30% of schizophrenic patients show persistent auditory hallucination despite appropriate pharmacotherapy.¹⁹⁾ It is interesting to note that the superior efficacy of clozapine may be explained by its preferential action in the cerebral cortex over the striatum.¹⁸⁾ Regional selectivity, although debatable, is certainly a key

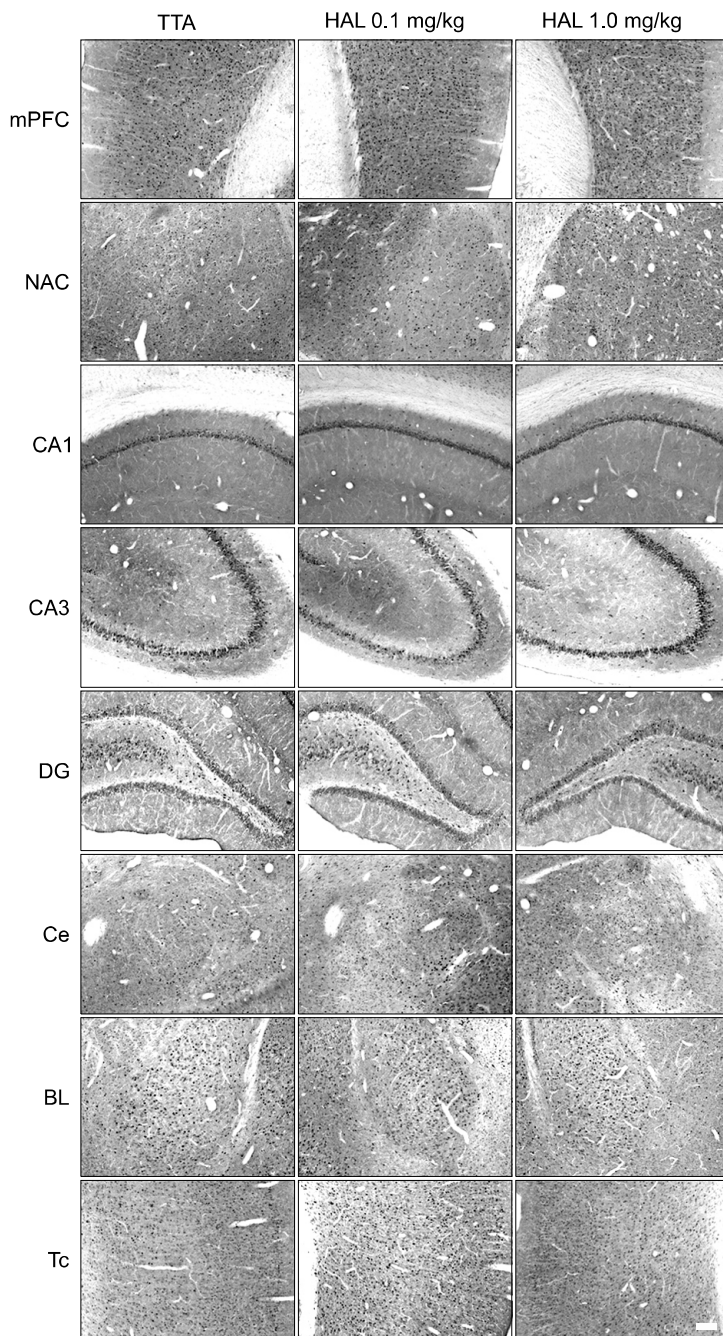


Fig. 2. Representative photomicrographs illustrating the number of Fos-positive neurons in the rat brain after single exposure to antipsychotic agents. TTA, tartaric acid; HAL, haloperidol-treated; mPFC, medial prefrontal cortex; NAC, nucleus accumbens; DG, dentate gyrus; Ce, central amygdala; BL, basolateral amygdala; Tc, temporal cortex. N=8 rats/group. Scale bar: 100 μ m.

concept that may be applicable in developing new antipsychotics. The amygdala is implicated in a variety of functions, including the expression of fear and the modulation of memory. It receives dopaminergic innervations from the ventral tegmental area²⁰ and is closely interconnected with the PFC and NAC.^{21,22} We observed a significant increase in FLI in the Ce and BL after all doses (1.0, 10 or 30 mg/kg) of aripiprazole, but a low dose of 0.1 mg/kg of haloperidol produced a significant increase in

FLI only in the Ce and BL. These findings regarding haloperidol treatment are consistent with the findings of other studies,^{23,24} implying that haloperidol has a limited effect in this area. On the other hand, clozapine (10-20 mg/kg)²³ and olanzapine (10 mg/kg)²⁴ both produce a significant increase in FLI in the Amyg. Therefore, these findings suggest that the induction of FLI in the Amyg, as in the PFC, might be a way to differentiate atypical from typical antipsychotics.

Several methodological considerations should be mentioned. In a preliminary experiment, we compared c-FOS expression between rats exposed to a habituation handling procedure for 3-4 days with naïve rats. The results showed significantly less induction of c-FOS expression in the rats exposed to handling. Hence, a habituation handling procedure for 4 days before the experiment was adopted as a standard procedure in the present study, which might be one of the strengths of our study. Another difference between our study and others is the time point for sacrificing animals after drug injection, which was 2 h in our study. We chose 2 h based on the fact that the protein product, Fos, peaks between 2 and 6 h after an acute challenge.²⁵⁾ However, there are many studies in which animals were sacrificed 4 h after acute drug administration. This difference may explain why we did not find that aripiprazole treatment preferentially affected FLI in the NAC-S over the NAC-C. The time point for sacrificing animals, therefore, should be considered in future studies.

In conclusion, these findings suggest that acute administration of aripiprazole or haloperidol significantly increases FLI in various brain areas, including the mPFC, NAC-S, NAC-C, CA1, CA3, DG, Ce, BL, and Tc. The differential action of aripiprazole but not haloperidol treatment on FLI in the mPFC and Amyg may be a good way to differentiate atypical from typical antipsychotics.

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