

Haloperidol, spiperone, pimozide and aripiprazole reduce intracellular dopamine content in PC12 cells and rat mesencephalic cultures: implication of inhibition of vesicular transport.

Takaaki Matsuo¹, Yasuhiko Izumi¹, Seiko Wakita¹, Toshiaki Kume¹, Yuki

Takada-Takatori², Hideyuki Sawada³ and Akinori Akaike¹

¹ Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan

² Department of Pharmacology, Faculty of Pharmaceutical Sciences, Doshisha Women's College, Kodo, Kyotanabe, Kyoto 610-0395, Japan

³ Department of Neurology and Clinical Research Center, Center for Neurological Diseases, Utano National Hospital, 8 Ondoyamacho, Narutaki, Ukyo-ku, Kyoto 616-8255, Japan

Corresponding author:

Akinori Akaike, Ph.D.

Department of Pharmacology, Graduate School of Pharmaceutical Sciences,
Kyoto University

46-29 Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto 606-8501, Japan

Tel.: +81-75-753-4550; fax: +81-75-753-4579

E-mail address: aakaike@pharm.kyoto-u.ac.jp

Abstract

Accumulating evidence suggests that antipsychotics affect dopamine release from dopaminergic neurons, but the precise mechanisms are not fully understood. Besides, there are few studies on the effects of antipsychotics on intracellular dopamine content. In this study, the effects of 8 antipsychotics on dopamine release and intracellular dopamine content in PC12 cells were investigated. Pretreatment with haloperidol, spiperone, pimozone, aripiprazole and risperidone markedly inhibited high potassium-evoked dopamine release. By contrast, pretreatment with chlorpromazine slightly increased high potassium-evoked dopamine release, while pretreatment with sulpiride and olanzapine had no effect. Haloperidol, spiperone, pimozone, chlorpromazine, aripiprazole and olanzapine evoked dopamine release, while sulpiride and risperidone had no effect. In addition, haloperidol, spiperone, pimozone, aripiprazole and risperidone reduced intracellular dopamine content in a concentration-dependent manner. These results suggest that the reduction in high potassium-evoked dopamine release by pretreatment with antipsychotics results from the reduction in vesicular dopamine content. Treatment with the 8 antipsychotics did not affect the expression of total or phosphorylated tyrosine hydroxylase. Instead, haloperidol, spiperone, pimozone and aripiprazole as well as reserpine transiently increased extracellular levels of dopamine metabolites. In addition, haloperidol, spiperone, pimozone, aripiprazole and risperidone reduced vesicular [³H]dopamine transport. These results suggest that the inhibition of vesicular dopamine transport by haloperidol, spiperone, pimozone and aripiprazole results in a reduction in vesicular dopamine content.

Indexwords: Antipsychotics; Dopamine release; Intracellular dopamine content;
Vesicular transport

1. Introduction

Recent studies using positron emission tomography (PET) or single photon emission computed tomography (SPECT) have clarified the pathophysiologic changes in presynaptic regions of dopaminergic neurons in schizophrenia. Higher [^{123}I]IBZM binding to dopamine D₂ receptors during depletion of endogenous dopamine in patients with schizophrenia than in healthy controls has suggested that occupancy of dopamine D₂ receptors by dopamine is increased (Abi-Dargham et al., 2000). In addition, increased striatal uptake of [^{18}F]DOPA or [^{11}C]DOPA and high displacement of [^{123}I]IBZM in response to amphetamine in patients with schizophrenia have suggested that presynaptic dopamine levels are increased (Abi-Dargham et al., 1998; Lindström et al., 1999; McGowan et al., 2004). These recent findings have indicated that presynaptic dopaminergic neurons themselves as well as postsynaptic dopamine receptors are important for an understanding of the pathophysiology of schizophrenia.

It has been reported that antipsychotics affected dopamine release, although their clinical efficacy may be attributed to the blocking of postsynaptic dopamine receptors. Haloperidol, spiperone and clozapine increased dopamine release in rat striatum *in vitro* or *in vivo* (Seeman and Lee, 1975; Dismukes and Mulder, 1977; Drew et al., 1990). These antipsychotics also inhibited the electrically-stimulated dopamine release from rat striatal slices (Seeman and Lee, 1975; Dismukes and Mulder, 1977) and it was suggested that the effect of haloperidol was not related to the interaction with dopamine D₂ receptors (Dismukes and Mulder, 1977). However, it has been reported that haloperidol and other antipsychotics increased the electrically-stimulated dopamine release by blocking dopamine autoreceptors in rat striatal slices (Farnebo and Hamberger, 1971; Dwoskin and Zahniser, 1986). Therefore, the effects of

antipsychotics on dopamine release are a matter of debate.

Since presynaptic dopamine levels are suggested to be increased in schizophrenia as described above, it is important to analyze the effects of antipsychotics on intracellular dopamine content as well as dopamine release. However, there are few studies on the effects of antipsychotics on intracellular dopamine content. Thus, in this study, we investigated intracellular dopamine content in rat adrenal pheochromocytoma PC12 cells and rat mesencephalic cultures after treatment with antipsychotics. PC12 cells express vesicular monoamine transporter (VMAT) 1, while dopaminergic neurons express VMAT 2 (Schuldiner, 1994). In spite of the difference, PC12 cells share many features of dopaminergic neurons relating to the synthesis, storage, release and metabolism of dopamine (Greene and Rein, 1977; Tuler et al., 1989). Therefore, we used PC12 cells as a dopaminergic neuronal model. We also used rat mesencephalic cultures because the cultures are known to contain dopaminergic neurons. Generally, antipsychotics are divided in two basic classes; typical and atypical. In this study, we used typical (haloperidol, spiperone, pimozide, chlorpromazine and sulpiride) and atypical (aripiprazole, risperidone and olanzapine) antipsychotics. All the antipsychotics have antagonistic activity for dopamine D₂ receptors, although aripiprazole is a dopamine D₂ receptor partial agonist and acts as an agonist on presynaptic autoreceptors (Kikuchi et al., 1995). Our findings revealed that haloperidol, spiperone, pimozide and aripiprazole reduced intracellular dopamine content and inhibited vesicular dopamine transport.

2. Materials and Methods

2.1. Reagents

Haloperidol, spiperone, pimozide, racemic sulpiride, risperidone, reserpine, adenosine 5'-triphosphate magnesium salt, digitonin and ascorbic acid were purchased from Sigma (St. Louis, MO). Chlorpromazine hydrochloride and pargyline hydrochloride were obtained from Nacalai Tesque (Kyoto, Japan). Olanzapine was purchased from Toronto Research Chemicals (Toronto, Canada). [7, 8-³H]dopamine was obtained from GE Healthcare (Buckinghamshire, UK). Aripiprazole was kindly provided by Otsuka Pharmaceutical Company (Tokyo, Japan).

2.2. Cell cultures

PC12 cells, obtained from American Type Culture Collection (Manassas, VA), were cultured on tissue-culture treated 100-mm cell culture dishes (Cat# 353003, BD Falcon, Franklin Lakes, NJ) in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and 10% horse serum as described previously (Kumar et al., 2003). Cell cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Rat mesencephalic cultures were established according to methods described previously (Izumi Y et al., 2009). Briefly, the ventral two-thirds of the mesencephalon were dissected from rat embryos on the 16th day of gestation. The tissues were then chemically and mechanically dissociated into single cell suspensions. Cells were plated onto 0.1% polyethyleneimine-coated plastic coverslips at a density of 1.3×10^5 cells/cm². Cultures were maintained in Eagle's minimum essential medium (EMEM) containing 10% fetal calf serum [1-4 days *in vitro* (DIV)] or horse serum [5-9 DIV]. We

changed fetal calf serum into horse serum during the latter term of cultivation in order to inhibit excessive glial proliferation. Cultures were also incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The animals were treated in accordance with the guidelines of the Kyoto University animal experimentation committee and of the Japanese Pharmacological Society.

2.3. Measurement of intracellular dopamine content

Intracellular dopamine content was measured by high performance liquid chromatography with electrochemical detection (HPLC-ED) as described previously (Izumi et al., 2008). PC12 cells were seeded into 35-mm cell culture dishes (BD Falcon) at a density of 8.3×10^4 cells/cm² and grown for 24 h, then exposed to drugs. Mesencephalic cultures were exposed to drugs at 8 DIV. To measure intracellular dopamine content after treatment with drugs for 24 h, cells were scraped, centrifuged and resuspended in extraction buffer (200 µL) (0.1 N perchloric acid containing 10 mM sodium disulfite and 1 mM EDTA). After sonication, lysates were centrifuged at 17,000 g for 30 min. The supernatants (2 µL) were analyzed by HPLC-ED. The detection limit in our system was 10 fmol dopamine. In this experiment, more than 100 fmol dopamine was used for analysis. Intracellular dopamine content in sham-treated cells was expressed as 100%. The HPLC system consisted of an automatic sample injector (Model 231, Gilson, Villiers-le-Bel, France), a pump (EP-300, Eicom, Kyoto, Japan), a degasser (DG-300, Eicom), a reverse-phase column (Eicompak CA-5ODS, inside diameter: 2.1 mm, length: 150 mm, Eicom), a column oven (ATC-300, Eicom) and an electrochemical detector (ECD-300, Eicom) with a working electrode versus an Ag/AgCl reference electrode. The working electrode was maintained at an oxidative

potential of +750 mV. The mobile phase consisted of 0.1 M acetate-citrate buffer (pH 3.7) containing 300 mg/L sodium octylsulfate, 5 mg/L EDTA-2Na and 16% methanol, and the flow rate was set at 0.23 mL/min.

2.4. Measurement of extracellular dopamine, 3, 4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) levels

To measure extracellular DOPAC and HVA levels after treatment with drugs for 24 h, PC12 cells were seeded into 35-mm cell culture dishes at a density of 8.3×10^4 cells/cm² and grown for 24 h, then exposed to drugs. After treatment with drugs for 24 h, the conditioned medium was added to one-tenth volume of extraction buffer ($\times 10$) and centrifuged. The supernatants (2 μ L) were analyzed by HPLC-ED. To measure extracellular dopamine, DOPAC and HVA levels after treatment with drugs for 1 h, PC12 cells were seeded into 48-well cell culture plates (BD Falcon) at a density of 1.1×10^5 cells/cm² and grown for 24 h, then exposed to drugs. Cells were treated with drugs in Krebs-Ringer-HEPES buffer (125 mM NaCl, 4.8 mM KCl, 25 mM HEPES, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 5.6 mM glucose and 2.2 mM CaCl₂, pH 7.4) for 1 h. Supernatants were added to one-tenth volume of extraction buffer ($\times 10$) and centrifuged. The supernatants (20 μ L) were analyzed by HPLC-ED.

2.5. Measurement of dopamine release in the presence of pargyline and ascorbic acid

PC12 cells were seeded into 48-well cell culture plates at a density of 1.1×10^5 cells/cm² and grown for 24 h. Cells were treated with drugs for 30 min or 1 h in Krebs-Ringer-HEPES buffer containing 10 μ M pargyline and 1 mM ascorbic acid. Pargyline and ascorbic acid were added in order to inhibit dopamine metabolism and to

prevent dopamine oxidation, respectively. Supernatants were added to one-tenth volume of extraction buffer ($\times 10$) and centrifuged. The supernatants (20-30 μL) were analyzed by HPLC-ED.

2.6. Western blotting

PC12 cells were seeded into 35-mm cell culture dishes at a density of 8.3×10^4 cells/ cm^2 and grown for 24 h, then exposed to drugs. After treatment with drugs for 24 h, cells were washed twice with cold Tris-buffered saline, harvested and lysed in buffer (200 μL) containing 20 mM Tris (pH 7.0), 25 mM β -glycerophosphate, 2 mM EGTA, 1% Triton-X-100, 1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 2 mM dithiothreitol and 1 mM vanadate on ice. The lysates were centrifuged at 17,000 g for 30 min at 4°C. The protein concentrations of the supernatant were normalized and mixed in equal amounts with sample buffer composed of 124 mM Tris (pH 6.8), 4% sodium dodecyl sulfate, 10% glycerol, 0.02% bromophenol blue and 4% 2-mercaptoethanol. After denaturation by boiling at 100°C for 5 min, samples (10 μg of protein) were loaded onto a sodium dodecyl sulfate-polyacrylamide gel, separated electrophoretically and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). The membrane was incubated for 1 h with 10 mM Tris-buffered saline (pH 7.6) containing 0.1% Tween 20 and 5% skim milk to block non-specific binding. Subsequently, the membrane was probed with anti-tyrosine hydroxylase (TH; Chemicon/Millipore, Temecula, CA), anti-phospho-Ser19 TH, anti-phospho-Ser31 TH and anti-phospho-Ser40 TH (Phosphosolutions, Aurora, CO) or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Ambion, Austin, TX) for 1 h and with horseradish peroxidase-conjugated secondary antibody (GE Healthcare) for

1 h. The membrane-bound secondary antibody was detected with an enhanced chemiluminescence detection system (ECL or ECL-Plus, GE Healthcare).

2.7. Measurement of vesicular [³H]dopamine transport in the presence of pargyline and ascorbic acid

Vesicular [³H]dopamine transport was determined as described previously (Höltje et al., 2000) with a slight modification (Izumi et al., 2008). After removing medium, cells from a confluent 100-mm dish were washed twice with 10 mM phosphate-buffered saline (pH 7.5) and harvested with potassium glutamate buffer (KG buffer; 150 mM potassium glutamate, 20 mM HEPES, 4 mM EGTA, 1 mM MgCl₂, 10 μM pargyline and 1 mM ascorbic acid, adjusted to pH 7.0 with KOH). Pargyline and ascorbic acid were added in order to inhibit dopamine metabolism and to prevent dopamine oxidation, respectively. The cell suspension was centrifuged and resuspended in KG buffer supplemented with 10 μM digitonin and incubated for 5 min at 37°C to selectively permeabilize the plasma membrane. Permeabilized cells were distributed into 30 tubes and washed by adding ice-cold KG buffer (500 μL) and spun down at 1,000 g for 2 min at 4°C. Uptake was started by adding KG buffer (100 μL) containing 5 mM Mg-ATP and 50 nM [³H]dopamine. Drugs to be tested were applied during this step. Incubation was performed for 30 min at 37°C and stopped by adding ice-cold KG buffer (700 μL), followed by a rapid centrifugation. After rinsing with KG buffer, the cell pellet was lysed in 0.4% Triton X-100 to measure radioactivity by scintillation counting. A non-specific value was determined using reserpine (10 μM) and subtracted from the values obtained experimentally.

2.8. *Statistics*

The statistical significance of the difference among three or more groups of individual data was analyzed by the one-way analysis of variance (ANOVA) and post hoc multiple comparison using Tukey's test. The statistical significance of the difference between two groups of individual data was analyzed by unpaired t test. Statistical significance was defined as $p < 0.05$. Data were expressed as the mean \pm S.E.M.

3. Results

3.1. Effects of antipsychotics on dopamine release in the presence of pargyline and ascorbic acid and intracellular dopamine content in PC12 cells

To investigate whether antipsychotics affect exocytotic dopamine release, the amount of extracellular dopamine released by high potassium was determined by HPLC-ED. In this experiment, we used pargyline and ascorbic acid to inhibit dopamine metabolism and to prevent dopamine oxidation, respectively. Treatment with pargyline and ascorbic acid for 1 h almost completely inhibited dopamine metabolism and retained extracellular dopamine (Fig. 1A-C). High potassium is known to induce depolarization and then evoke a calcium-dependent release of dopamine. We confirmed that the high potassium-evoked release did not occur in calcium-free buffer (sham, $100 \pm 23.4\%$; KCl (60 mM), $119.3 \pm 14.8\%$). After exposure to antipsychotics (1 μ M) for 24 h, PC12 cells were stimulated with KCl (60 mM) for 30 min. Haloperidol, spiperone, pimozide, aripiprazole and risperidone markedly reduced high potassium-evoked dopamine release. Chlorpromazine slightly increased, but sulpiride and olanzapine did not affect high potassium-evoked dopamine release (Fig. 1D). To investigate whether antipsychotics affect spontaneous dopamine release, the amount of extracellular dopamine released by the treatment with antipsychotics (1 μ M) for 1 h was determined by HPLC-ED. Haloperidol, spiperone, pimozide, chlorpromazine, aripiprazole and olanzapine evoked dopamine release, whereas sulpiride and risperidone had no effect (Fig. 1E). Next, we examined the effects of antipsychotics on intracellular dopamine content in PC12 cells. Cells were exposed to drugs (1 μ M) for 24 h, and then intracellular dopamine content was measured by HPLC-ED. Haloperidol, spiperone, pimozide and aripiprazole markedly reduced intracellular dopamine content and

risperidone did so modestly. Chlorpromazine increased intracellular dopamine content slightly, while sulpiride and olanzapine had no effect (Fig. 1F).

To examine the concentration dependency of the effects of these drugs on intracellular dopamine content, PC12 cells were treated with various concentrations of drugs for 24 h. Haloperidol, spiperone, pimozide, aripiprazole and risperidone reduced intracellular dopamine content in a concentration-dependent manner, while chlorpromazine increased intracellular dopamine content in a bell-shaped concentration-dependent manner (Fig. 2).

3.2. Effects of antipsychotics on intracellular dopamine content in primary mesencephalic dopaminergic neurons

To confirm the effect of antipsychotics on intracellular dopamine content, we used primary mesencephalic cultures, which contain dopaminergic neurons. After the exposure of mesencephalic cultures to drugs (1 μ M) for 24 h, intracellular dopamine content was measured by HPLC-ED. As shown in Fig. 3, haloperidol, spiperone, pimozide, aripiprazole and risperidone reduced intracellular dopamine content significantly, while chlorpromazine, sulpiride and olanzapine had no effect. The drugs which reduced intracellular dopamine content in PC12 cells were the same as those that did so in mesencephalic cultures.

3.3. Effects of antipsychotics on the expression of tyrosine hydroxylase in PC12 cells

Next, we examined the mechanisms behind the reduction in intracellular dopamine content. TH is a rate-limiting enzyme in dopamine synthesis and the phosphorylation of TH at serine (Ser) residues, Ser19, Ser31 and Ser40, is known to

increase its activity (Dunkley et al., 2004). Total and phosphorylated TH protein levels were examined to investigate whether the reduction in intracellular dopamine content results from inhibition of dopamine synthesis. Exposure to antipsychotics (1 μ M) for 24 h did not affect total or phosphorylated TH protein levels (Fig. 4).

3.4. Effects of antipsychotics on the extracellular DOPAC and HVA levels in PC12 cells

It is known that dopamine is metabolized to DOPAC and HVA, which are mainly present extracellularly. To investigate whether the reduction in intracellular dopamine content results from the facilitation of dopamine metabolism, extracellular DOPAC and HVA levels in PC12 cells after exposure to antipsychotics (1 μ M) for 24 h were measured by HPLC-ED. Neither extracellular DOPAC nor extracellular HVA levels were changed (Fig. 5).

We also measured extracellular dopamine, DOPAC and HVA levels in PC12 cells after exposure to antipsychotics or reserpine (1 μ M) for 1 h by HPLC-ED. Haloperidol, spiperone, pimozide and aripiprazole as well as reserpine increased extracellular DOPAC and HVA levels, though pimozide and aripiprazole increased extracellular dopamine levels in addition to extracellular dopamine metabolites. Chlorpromazine markedly and olanzapine slightly increased only extracellular dopamine levels, while sulpiride and risperidone had no effect on extracellular levels of dopamine and dopamine metabolites (Fig. 6).

3.5. Effects of antipsychotics on vesicular [³H]dopamine transport in the presence of pargyline and ascorbic acid in PC12 cells.

To investigate whether antipsychotics reduce intracellular dopamine content

via a reserpine-like effect, the effect of antipsychotics on vesicular dopamine transport was examined. In this experiment, pargyline and ascorbic acid were added in order to inhibit dopamine metabolism and to prevent dopamine oxidation, respectively. In digitonin-permeabilized cells, the amount of [³H]dopamine taken up into vesicles for 30 min in the presence of antipsychotics (10 μM) was measured. Haloperidol, spiperone, pimozide, aripiprazole and risperidone inhibited the incorporation of [³H]dopamine into vesicles. On the other hand, chlorpromazine, sulpiride and olanzapine had no effect (Fig. 7).

4. Discussion

In this study, we examined the effects of antipsychotics on dopamine release in the presence of pargyline and ascorbic acid. Pretreatment with haloperidol, spiperone, pimozide, aripiprazole and risperidone reduced high potassium-evoked dopamine release. In addition, haloperidol, spiperone, pimozide, aripiprazole and risperidone also reduced intracellular dopamine content in a concentration-dependent manner in PC12 cells. As high potassium-evoked dopamine release was attributed to calcium-dependent exocytosis, it is suggested that these antipsychotics may reduce vesicular dopamine content. Furthermore, we confirmed that the same drugs decreased intracellular dopamine content in mesencephalic cultures. Therefore, it is suggested that the effects of antipsychotics on intracellular dopamine content are not restricted to PC12 cells. Sulpiride and olanzapine had no effect on high potassium-evoked dopamine release or intracellular dopamine content. Chlorpromazine slightly increased the high potassium-evoked dopamine release and intracellular dopamine content in PC12 cells. However, the concentration at which chlorpromazine significantly increased intracellular dopamine content changed between 0.3 μ M and 1 μ M. The variation in the concentration might be brought about by a slight difference in the experimental conditions.

We used antipsychotics at a concentration of 1 μ M, which was much higher than K_i values for dopamine D_2 receptors (Burstein et al., 2005). Therefore, it was considered that all antipsychotics tested in this study sufficiently antagonize dopamine D_2 receptors. However, chlorpromazine, sulpiride and olanzapine did not reduce intracellular dopamine content, while haloperidol, spiperone, pimozide, aripiprazole and risperidone reduced intracellular dopamine content in a concentration-dependent

manner. Furthermore, we have reported that dopamine receptor agonists as well as antagonists reduced intracellular dopamine content in PC12 cells (Izumi et al., 2008). Taken together, we considered that dopamine D₂ receptors are not necessarily required for the reduction of intracellular dopamine content by antipsychotics.

We investigated the mechanisms underlying the reduction of intracellular dopamine content by antipsychotics. The present results showed that antipsychotics had no effect on the expression of total TH or phosphorylated TH, indicating that the reduction is not brought about by the inhibition of dopamine synthesis. Inhibition of vesicular dopamine transport is thought to be another mechanism behind the reduction of intracellular dopamine content. It is known that reserpine, an inhibitor of VMAT, causes the depletion of intracellular dopamine. Furthermore, it has been reported that reserpine increased catecholamine release (Mahata et al., 1996) and we confirmed that reserpine evoked dopamine release in our experiments (data not shown). Haloperidol, spiperone, pimozide and aripiprazole, which reduced intracellular dopamine content, as well as reserpine, increased dopamine release in the presence of pargyline and ascorbic acid. Risperidone reduced intracellular dopamine content, but did not significantly increase dopamine release. Since the reduction in intracellular dopamine content caused by risperidone was less than that caused by haloperidol, spiperone, pimozide or aripiprazole, the change in dopamine release caused by risperidone might be undetectable. In addition, reserpine increases extracellular DOPAC and HVA levels (Anden et al., 1964; Roffler-Tarlov et al., 1971) because cytosolic dopamine which is not incorporated into vesicles by reserpine is metabolized and transported extracellularly (Roffler-Tarlov et al., 1971). In the present study, treatment with haloperidol, spiperone, pimozide and aripiprazole as well as reserpine for 1 h increased

extracellular DOPAC and HVA levels. Therefore, it is suggested that haloperidol, spiperone, pimozide and aripiprazole have a reserpine-like effect. Although treatment with haloperidol, spiperone, pimozide or aripiprazole for 1 h increased extracellular DOPAC and HVA levels significantly, these antipsychotics did not change the total amount of extracellular DOPAC or HVA for 24 h. It is suggested that the transient change in 1 h was hidden because the increase in extracellular dopamine metabolites for 1 h was very low compared to the total amounts of metabolites released extracellularly for 24 h. Treatment with pimozide, chlorpromazine, aripiprazole and olanzapine for 1 h increased extracellular dopamine levels in the presence or absence of pargyline and ascorbic acid. They might have additional mechanisms for dopamine release.

Haloperidol, spiperone, pimozide and aripiprazole, which reduced intracellular dopamine content and increased extracellular DOPAC and HVA levels, inhibited vesicular [^3H]dopamine transport in the presence of pargyline and ascorbic acid. These results suggest that the cytosolic dopamine which is not incorporated into vesicles is metabolized, and then released extracellularly, leading to the reduction of intracellular dopamine content. Previously, it has been reported that haloperidol had reserpine-like effects (Delanoy and Dunn, 1982) and that spiperone inhibited the transport of serotonin into membrane vesicles isolated from platelet dense granules (Dannies et al., 1984). Haloperidol inhibited vesicular transport and the formation of the pH gradient of synaptic vesicles, although haloperidol at concentrations that inhibited vesicular transport did not dissipate the pH gradient (Moriyama et al., 1993; Yasumoto et al., 2009). In addition, it has been reported that pramipexole and ketanserin, an antagonist of serotonergic receptors, bound directly to VMAT (Darchen

et al., 1988; Izumi et al., 2008). Taking these reports into consideration, the inhibition of vesicular transport by antipsychotics may derive from a reduction of the vesicular pH gradient or from direct inhibition of transporter activity. As described in the Introduction, VMAT 1 is expressed in PC12 cells, while VMAT 2 is expressed in the brain (Schuldiner, 1994). Regardless of the difference in the isoforms, in our experiments, the reduction of intracellular dopamine content by antipsychotics was observed in both PC12 cells and mesencephalic cultures. These results suggest that the difference in VMAT isoforms does not influence the inhibition of vesicular transport by antipsychotics. Since VMAT is also responsible for the transport of serotonin, norepinephrine and epinephrine into vesicles, haloperidol, spiperone, pimozide and aripiprazole are likely to affect vesicular transport of the other monoamines as well as dopamine. In fact, a previous report demonstrated that spiperone inhibited the transport of serotonin into membrane vesicles (Dannies et al., 1984).

As described above, presynaptic dopamine content is suggested to be increased in patients with schizophrenia (Abi-Dargham et al., 1998; Lindström et al., 1999; McGowan et al., 2004). It is thought that the increase in presynaptic dopamine content mainly represents the increase in vesicular dopamine content because most of the intracellular dopamine in dopamine neurons is accumulated into synaptic vesicles (Lyon et al., 2009). However, since it is known that catecholamines leak from vesicles into cytosol (Floor et al., 1995; Eisenhofer et al., 2004), cytosolic dopamine may also be increased in schizophrenia. Excessive cytosolic dopamine could be harmful to dopaminergic neurons because dopamine was easily oxidized and produced reactive oxygen species and oxidized intermediates (Stokes et al., 1999). These factors inactivated tyrosine hydroxylase, impaired the dopamine transporter and inhibited

mitochondrial NAD(P)H dehydrogenase (Ben-Shachar et al., 1995; Kuhn et al., 1999; Whitehead et al., 2001). All the antipsychotics used in this study are effective against schizophrenia by inhibiting dopamine-mediated signal transduction in postsynaptic neurons. In addition, it is possible that haloperidol, spiperone, pimozide, aripiprazole and risperidone, which reduced intracellular dopamine content in the present study, suppress the dysfunction of dopaminergic neurons.

In summary, haloperidol, spiperone, pimozide and aripiprazole reduced exocytotic dopamine release, intracellular dopamine content and vesicular dopamine transport. These findings suggest that the inhibition of vesicular transport by these antipsychotics induces the decrease in vesicular dopamine content. Further studies will be required to elucidate the mechanism underlying the inhibition of vesicular transport by these antipsychotics.

Acknowledgements

This work was supported by grants-in-aid for Scientific Research from the Japan Society for the Promotion of Science and from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

- Abi-Dargham, A., Gil, R., Krystal, J., Baldwin, R.M., Seibyl, J.P., Bowers, M., van Dyck, C.H., Charney, D.S., Innis, R.B., Laruelle, M., 1998. Increased striatal dopamine transmission in schizophrenia: confirmation in a second cohort. *Am. J. Psychiatry* 155, 761-767.
- Abi-Dargham, A., Rodenhiser, J., Printz, D., Zea-Ponce, Y., Gil, R., Kegeles, L.S., Weiss, R., Cooper, T.B., Mann, J.J., Van Heertum, R.L., Gorman, J.M., Laruelle, M., 2000. Increased baseline occupancy of D₂ receptors by dopamine in schizophrenia. *Proc. Natl. Acad. Sci. U.S.A.* 97, 8104-8109.
- Anden, N.E., Roos, B.E., Werdinius, B., 1964. Effects of chlorpromazine, haloperidol and reserpine on the levels of phenolic acids in rabbit corpus striatum. *Life Sci.* 3, 149-158.
- Ben-Shachar, D., Zuk, R., Glinka, Y., 1995. Dopamine neurotoxicity: inhibition of mitochondrial respiration. *J. Neurochem.* 64, 718-723.
- Burstein, E.S., Ma, J., Wong, S., Gao, Y., Pham, E., Knapp, A.E., Nash, N.R., Olsson, R., Davis, R.E., Hacksell, U., Weiner, D.M., Brann, M.R., 2005. Intrinsic efficacy of antipsychotics at human D₂, D₃, and D₄ dopamine receptors: identification of the clozapine metabolite N-desmethylozapine as a D₂/D₃ partial agonist. *J. Pharmacol. Exp. Ther.* 315, 1278-1287.
- Dannies, P.S., Rudnick, M.S., Fishkes, H., Rudnick, G., 1984. Spiperone: evidence for uptake into secretory granules. *Proc. Natl. Acad. Sci. U.S.A.* 81, 1867-1870.
- Darchen, F., Scherman, D., Laduron, P.M., Henry, J.P., 1988. Ketanserin binds to the monoamine transporter of chromaffin granules and of synaptic vesicles. *Mol. Pharmacol.* 33, 672-677.
- Delanoy, R.L., Dunn, A.J., 1982. Effects of haloperidol and apomorphine on catecholamine metabolism in brain slices. Reserpine-like effects of haloperidol.

- Biochem. Pharmacol. 31, 3297-3305.
- Dismukes, K., Mulder, A.H., 1977. Effects of neuroleptics on release of 3H-dopamine from slices of rat corpus striatum. *Naunyn Schmiedebergs Arch. Pharmacol.* 297, 23-29.
- Drew, K.L., O'Connor, W.T., Kehr, J., Ungerstedt, U., 1990. Regional specific effects of clozapine and haloperidol on GABA and dopamine release in rat basal ganglia. *Eur. J. Pharmacol.* 187, 385-397.
- Dunkley, P.R., Bobrovskaya, L., Graham, M.E., von Nagy-Felsobuki, E.I., Dickson, P.W., 2004. Tyrosine hydroxylase phosphorylation: regulation and consequences. *J. Neurochem.* 91, 1025-1043.
- Dwoskin, L.P., Zahniser, N.R., 1986. Robust modulation of [3H]dopamine release from rat striatal slices by D-2 dopamine receptors. *J. Pharmacol. Exp. Ther.* 239, 442-453.
- Eisenhofer, G., Kopin, I.J., Goldstein, D.S., 2004. Leaky catecholamine stores: undue waste or a stress response coping mechanism? *Ann. N. Y. Acad. Sci.* 1018, 224-230.
- Farnebo, L.O., Hamberger, B., 1971. Drug-induced changes in the release of 3 H-monoamines from field stimulated rat brain slices. *Acta Physiol. Scand. Suppl.* 371, 35-44.
- Floor, E., Leventhal, P.S., Wang, Y., Meng, L., Chen, W., 1995. Dynamic storage of dopamine in rat brain synaptic vesicles in vitro. *J. Neurochem.* 64, 689-699.
- Greene, L.A., Rein, G., 1977. Release, storage and uptake of catecholamines by a clonal cell line of nerve growth factor (NGF) responsive pheo-chromocytoma cells. *Brain Res.* 129, 247-263.
- Höltje, M., von Jagow, B., Pahner, I., Lautenschlager, M., Hörtnagl, H., Nürnberg, B., Jahn, R., Ahnert-Hilger, G., 2000. The neuronal monoamine transporter VMAT2 is regulated by the trimeric GTPase Go₂. *J. Neurosci.* 20, 2131-2141.

- Izumi, Y., Yamamoto, N., Kume, T., Katsuki, H., Sawada, H., Akaike, A., 2008.
Regulation of intracellular dopamine levels by dopaminergic drugs: involvement of vesicular monoamine transporter. *Eur. J. Pharmacol.* 582, 52-61.
- Izumi, Y., Yamamoto, N., Matsuo, T., Wakita, S., Takeuchi, H., Kume, T., Katsuki, H., Sawada, H., Akaike, A., 2009. Vulnerability to glutamate toxicity of dopaminergic neurons is dependent on endogenous dopamine and MAPK activation. *J. Neurochem.* 110, 745-755.
- Kikuchi, T., Tottori, K., Uwahodo, Y., Hirose, T., Miwa, T., Oshiro, Y., Morita, S., 1995.
7-(4-[4-(2,3-Dichlorophenyl)-1-piperazinyl]butyloxy)-3,4-dihydro-2(1H)-quinolinone (OPC-14597), a new putative antipsychotic drug with both presynaptic dopamine autoreceptor agonistic activity and postsynaptic D2 receptor antagonistic activity. *J. Pharmacol. Exp. Ther.* 274, 329-336.
- Kuhn, D.M., Arthur, R.E. Jr., Thomas, D.M., Elferink, L.A., 1999. Tyrosine hydroxylase is inactivated by catechol-quinones and converted to a redox-cycling quinoprotein: possible relevance to Parkinson's disease. *J. Neurochem.* 73, 1309-1317.
- Kumar, G.K., Kim, D.K., Lee, M.S., Ramachandran, R., Prabhakar, N.R., 2003.
Activation of tyrosine hydroxylase by intermittent hypoxia: involvement of serine phosphorylation. *J. Appl. Physiol.* 95, 536-544.
- Lindström, L.H., Gefvert, O., Hagberg, G., Lundberg, T., Bergström, M., Hartvig, P., Långström, B., 1999. Increased dopamine synthesis rate in medial prefrontal cortex and striatum in schizophrenia indicated by L-(β - ^{11}C) DOPA and PET. *Biol. Psychiatry* 46, 681-688.
- Lyon, G.J., Abi-Dargham, A., Moore, H., Lieberman, J.A., Javitch, J.A., Sulzer, D., 2009. Presynaptic Regulation of Dopamine Transmission in Schizophrenia.

- Schizophr. Bull. doi:10.1093/schbul/sbp010.
- Mahata, M., Mahata, S.K., Parmer, R.J., O'Connor, D.T., 1996. Vesicular monoamine transport inhibitors. Novel action at calcium channels to prevent catecholamine secretion. *Hypertension* 28, 414-420.
- McGowan, S., Lawrence, A.D., Sales, T., Quedsted, D., Grasby, P., 2004. Presynaptic dopaminergic dysfunction in schizophrenia: a positron emission tomographic [^{18}F]fluorodopa study. *Arch. Gen. Psychiatry* 61, 134-142.
- Moriyama, Y., Tsai, H.L., Futai, M., 1993. Energy-dependent accumulation of neuron blockers causes selective inhibition of neurotransmitter uptake by brain synaptic vesicles. *Arch. Biochem. Biophys.* 305, 278-281.
- Roffler-Tarlov, S., Sharman, D.F., Tegerdine, P., 1971. 3,4-dihydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic acid in the mouse striatum: a reflection of intra- and extra-neuronal metabolism of dopamine? *Br. J. Pharmacol.* 42, 343-351.
- Schuldiner, S., 1994. A molecular glimpse of vesicular monoamine transporters. *J. Neurochem.* 62, 2067-2078.
- Seeman, P., Lee, T., 1975. Antipsychotic drugs: direct correlation between clinical potency and presynaptic action on dopamine neurons. *Science* 188, 1217-1219.
- Stokes, A.H., Hastings, T.G., Vrana, K.E., 1999. Cytotoxic and genotoxic potential of dopamine. *J. Neurosci. Res.* 55, 659-665.
- Tuler, S.M., Hazen, A.A., Bowen, J.M., 1989. Release and metabolism of dopamine in a clonal line of pheochromocytoma (PC12) cells exposed to fenthion. *Fundam. Appl. Toxicol.* 13, 484-492.
- Whitehead, R.E., Ferrer, J.V., Javitch, J.A., Justice, J.B., 2001. Reaction of oxidized dopamine with endogenous cysteine residues in the human dopamine transporter. *J.*

Neurochem. 76, 1242-1251.

Yasumoto, S., Tamura, K., Karasawa, J., Hasegawa, R., Ikeda, K., Yamamoto, T.,
Yamamoto, H., 2009. Inhibitory effect of selective serotonin reuptake inhibitors on
the vesicular monoamine transporter 2. Neurosci. Lett. 454, 229-232.

Figure legends

Fig. 1 Effects of antipsychotics on dopamine release in the presence of pargyline and ascorbic acid and intracellular dopamine content in PC12 cells. A-C: Cells were incubated with Krebs-Ringer-HEPES buffer for 1 h in the presence of pargyline (10 μ M) and ascorbic acid (1 mM). The extracellular dopamine (A), DOPAC (B) and HVA (C) levels were determined by HPLC-ED. Data shown are mean values \pm S.E.M. ($n=3$) from a representative experiment. $^{##}p < 0.01$, $^{###}p < 0.001$, compared with control. D: Cells were pretreated with 8 kinds of drugs (1 μ M) for 24 h. After a washout, cells were treated with KCl (60 mM) for 30 min in the presence of pargyline (10 μ M) and ascorbic acid (1 mM) and then the extracellular dopamine levels were measured by HPLC-ED. Pargyline and ascorbic acid were added in order to inhibit dopamine metabolism and to prevent dopamine oxidation, respectively. Data shown are mean values \pm S.E.M. ($n=3$) from a representative experiment. $^{###}p < 0.001$, compared with KCl alone. E: Cells were treated with antipsychotics (1 μ M) for 1 h in the presence of pargyline (10 μ M) and ascorbic acid (1 mM). The extracellular dopamine levels were determined by HPLC-ED. Data shown are mean values \pm S.E.M. ($n=4$) from a representative experiment. $^{##}p < 0.01$, $^{###}p < 0.001$, compared with sham. F: Cells were treated with antipsychotics (1 μ M) for 24 h and then the intracellular dopamine content was measured by HPLC-ED. Data shown are mean values \pm S.E.M. ($n=3$) from a representative experiment. $^{\#}p < 0.05$, $^{###}p < 0.001$, compared with sham. Each experiment was repeated three times.

Fig. 2 Concentration-dependent effects of antipsychotics on intracellular dopamine content in PC12 cells. A-H: Cells were treated with various concentrations of drugs

(0.1-3 μ M) for 24 h and then the intracellular dopamine content was measured by HPLC-ED. Data shown are mean values \pm S.E.M. ($n=3$) from a representative experiment. Each experiment was repeated three times. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with sham.

Fig. 3 Effects of antipsychotics on intracellular dopamine content in primary mesencephalic cultures. A: Cultures were treated with typical antipsychotics (1 μ M) for 24 h and then the intracellular dopamine content was measured by HPLC-ED. B: Cultures were treated with atypical antipsychotics (1 μ M) for 24 h and then the intracellular dopamine content was measured by HPLC-ED. Data shown are mean values \pm S.E.M. ($n=3$) from a representative experiment. Each experiment was repeated three times. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with sham.

Fig. 4 Effects of antipsychotics on total and phosphorylated TH expression in PC12 cells. Cells were treated with antipsychotics (1 μ M) for 24 h. Total TH (~60 kDa), phosphorylated TH (p -Ser19, p -Ser31 and p -Ser40 TH; ~60 kDa) and GAPDH (36 kDa) protein levels were analyzed by Western blotting with the respective antibody. The experiment was repeated three times.

Fig. 5 Effects of treatment with antipsychotics for 24 h on extracellular DOPAC and HVA levels in PC12 cells. Cells were treated with antipsychotics (1 μ M) for 24 h. The extracellular DOPAC and HVA levels were measured by HPLC-ED. Data shown are mean values \pm S.E.M. ($n=3$) from a representative experiment. Each experiment was repeated three times.

Fig. 6 Effects of treatment with antipsychotics for 1 h on extracellular dopamine, DOPAC and HVA levels in PC12 cells. Cells were treated with antipsychotics (1 μ M) or reserpine (1 μ M) for 1 h. The extracellular dopamine (A), DOPAC (B) and HVA (C) levels were measured by HPLC-ED. Data shown are mean values \pm S.E.M. ($n=4$) from a representative experiment. Each experiment was repeated three times. $^{##}p < 0.01$, $^{###}p < 0.001$, compared with sham.

Fig. 7 Effects of antipsychotics on vesicular [3 H]dopamine transport in the presence of pargyline and ascorbic acid in PC12 cells. Pargyline (10 μ M) and ascorbic acid (1 mM) were added in order to inhibit dopamine metabolism and to prevent dopamine oxidation, respectively. Permeabilized PC12 cells were loaded with [3 H]dopamine for 30 min in the presence of antipsychotics (10 μ M). Data shown are mean values \pm S.E.M. ($n=3$) from a representative experiment. The experiment was repeated three times. $^{\#}p < 0.05$, $^{###}p < 0.001$, compared with sham.

Figure 1
[Click here to download high resolution image](#)

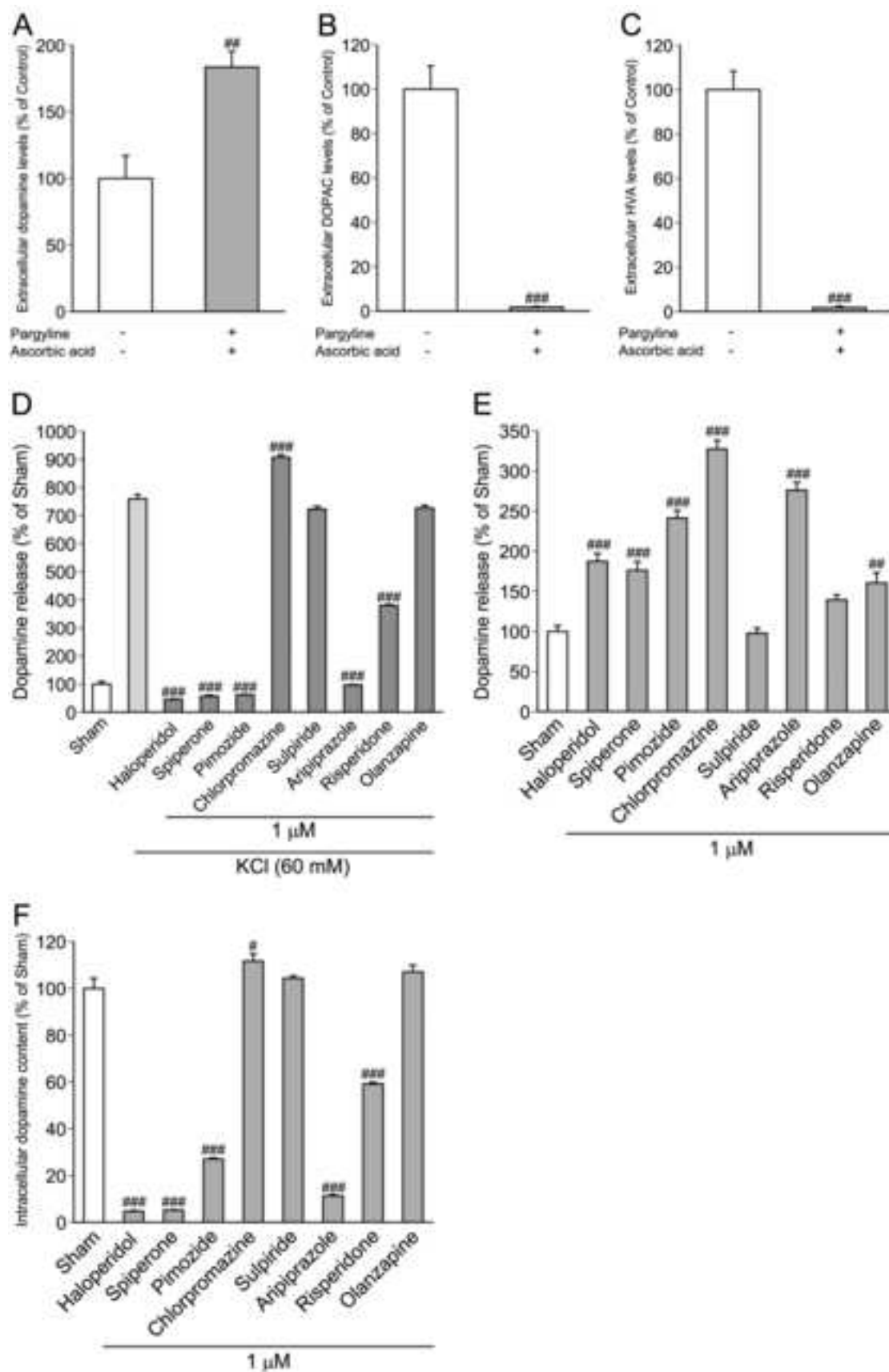


Figure 1 Matsuo et al.

Figure 2
[Click here to download high resolution image](#)

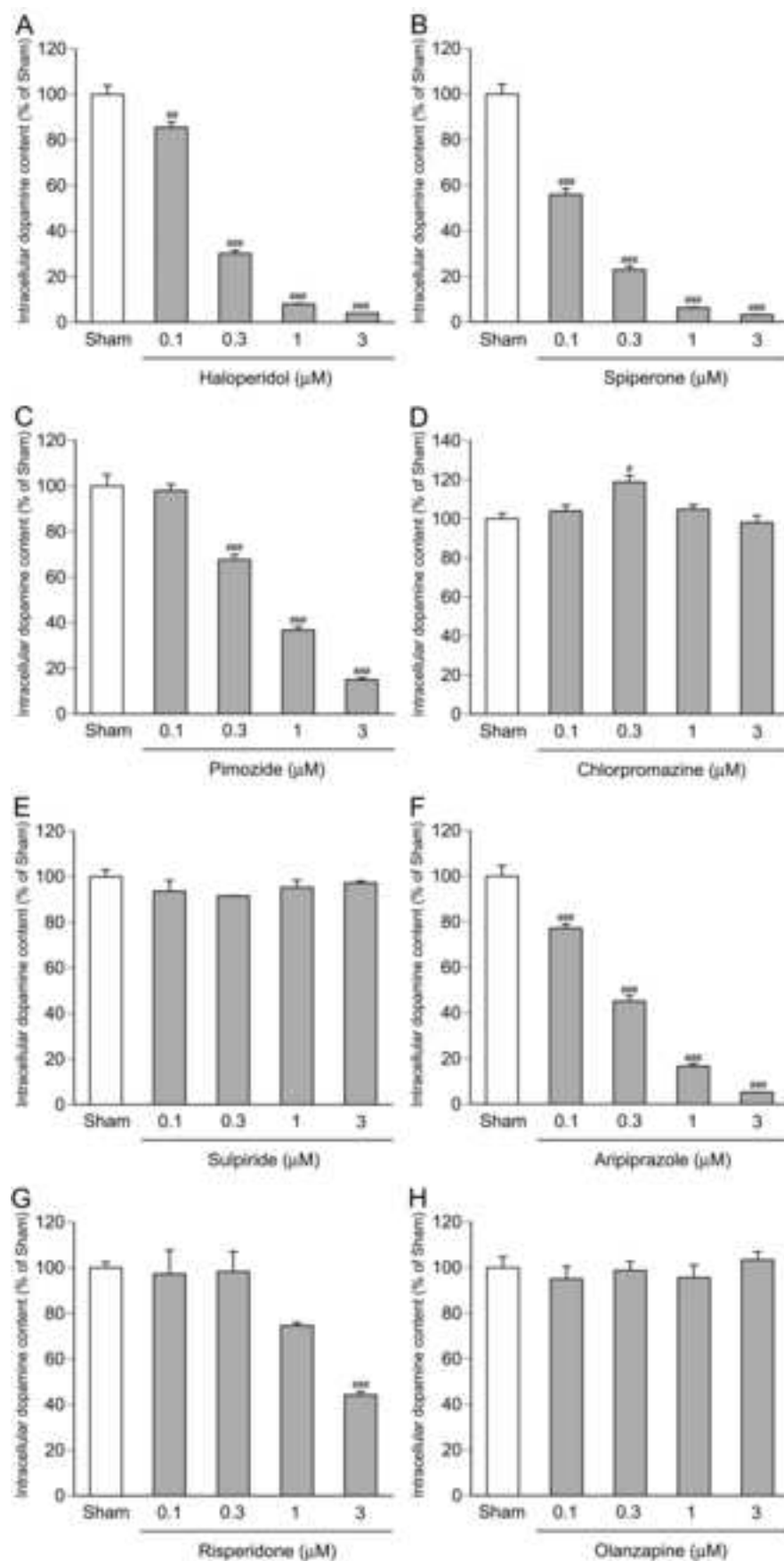


Figure 2 Matsuo et al.

Figure 3
[Click here to download high resolution image](#)

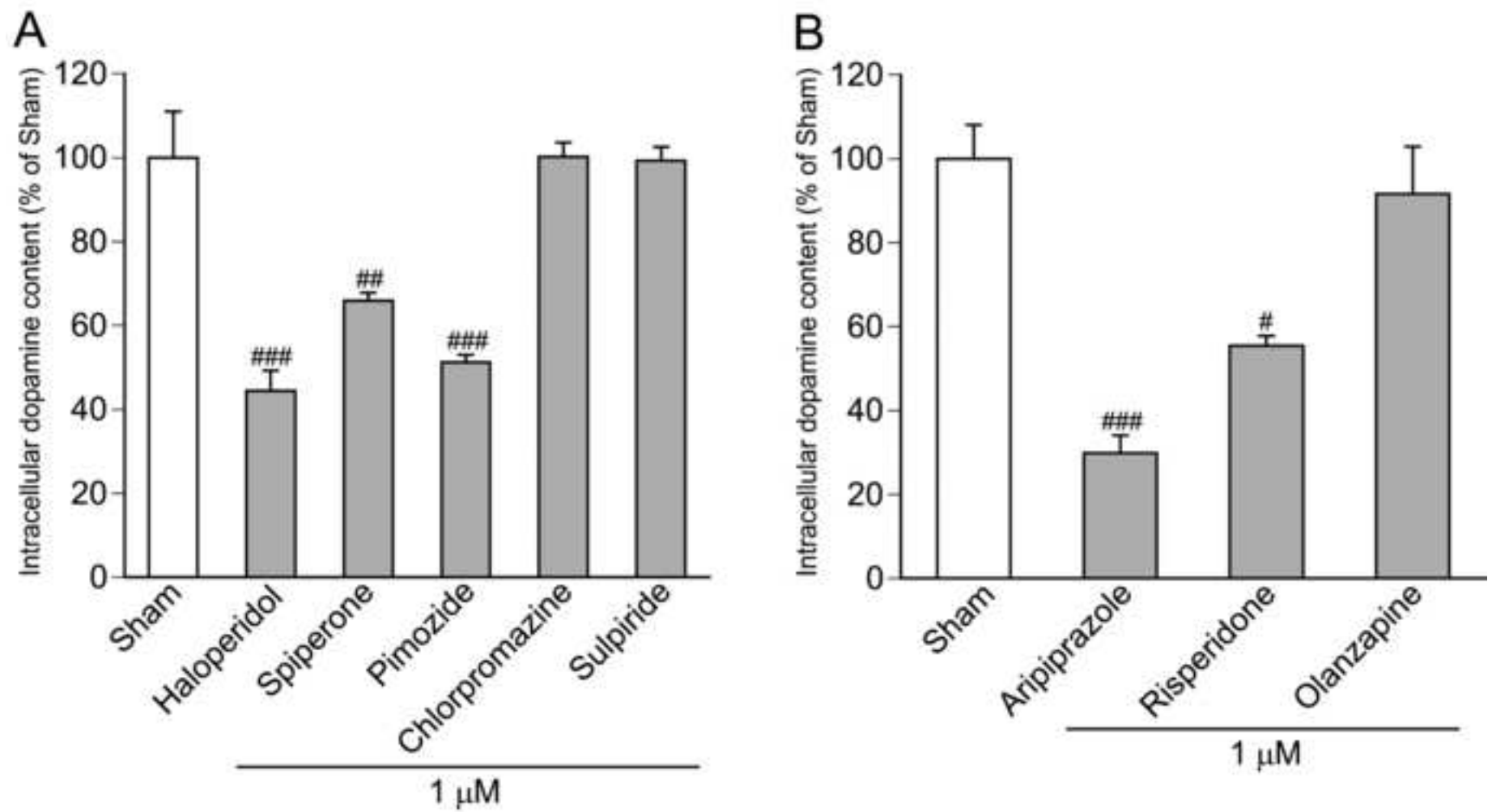


Figure 3 Matsuo et al.

Figure 4
[Click here to download high resolution image](#)

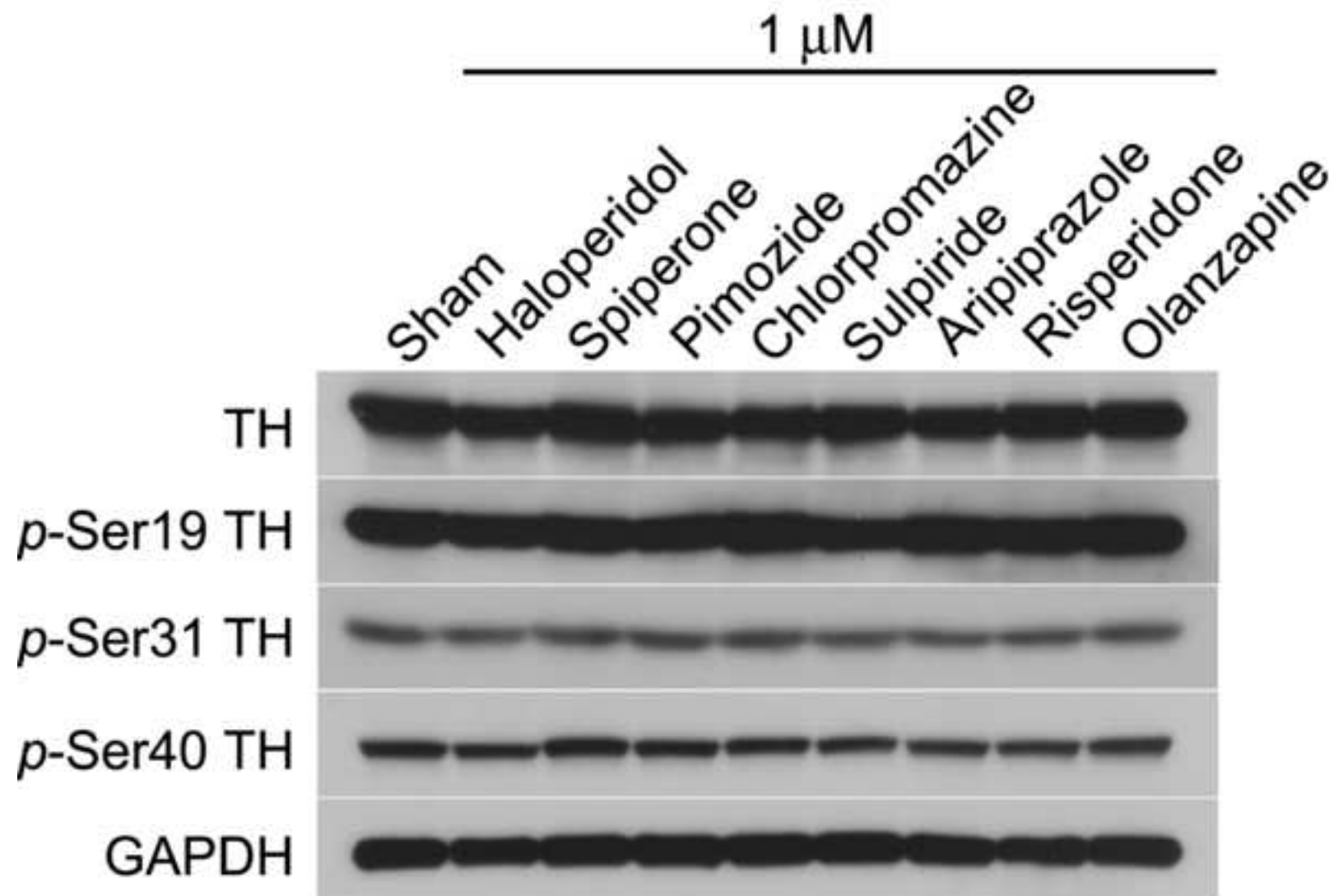


Figure 4 Matsuo et al.

Figure 5
[Click here to download high resolution image](#)

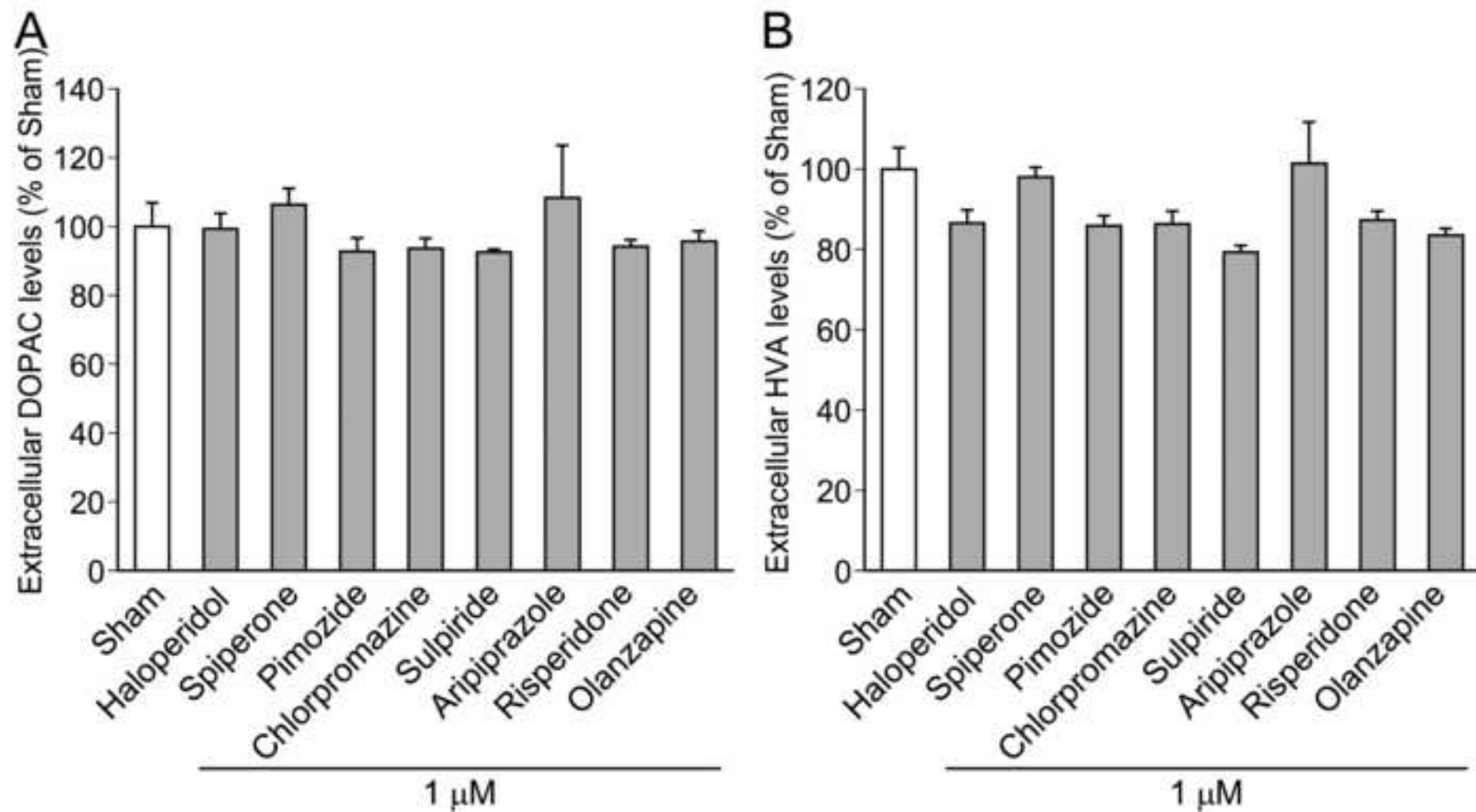


Figure 5 Matsuo et al.

Figure 6
[Click here to download high resolution image](#)

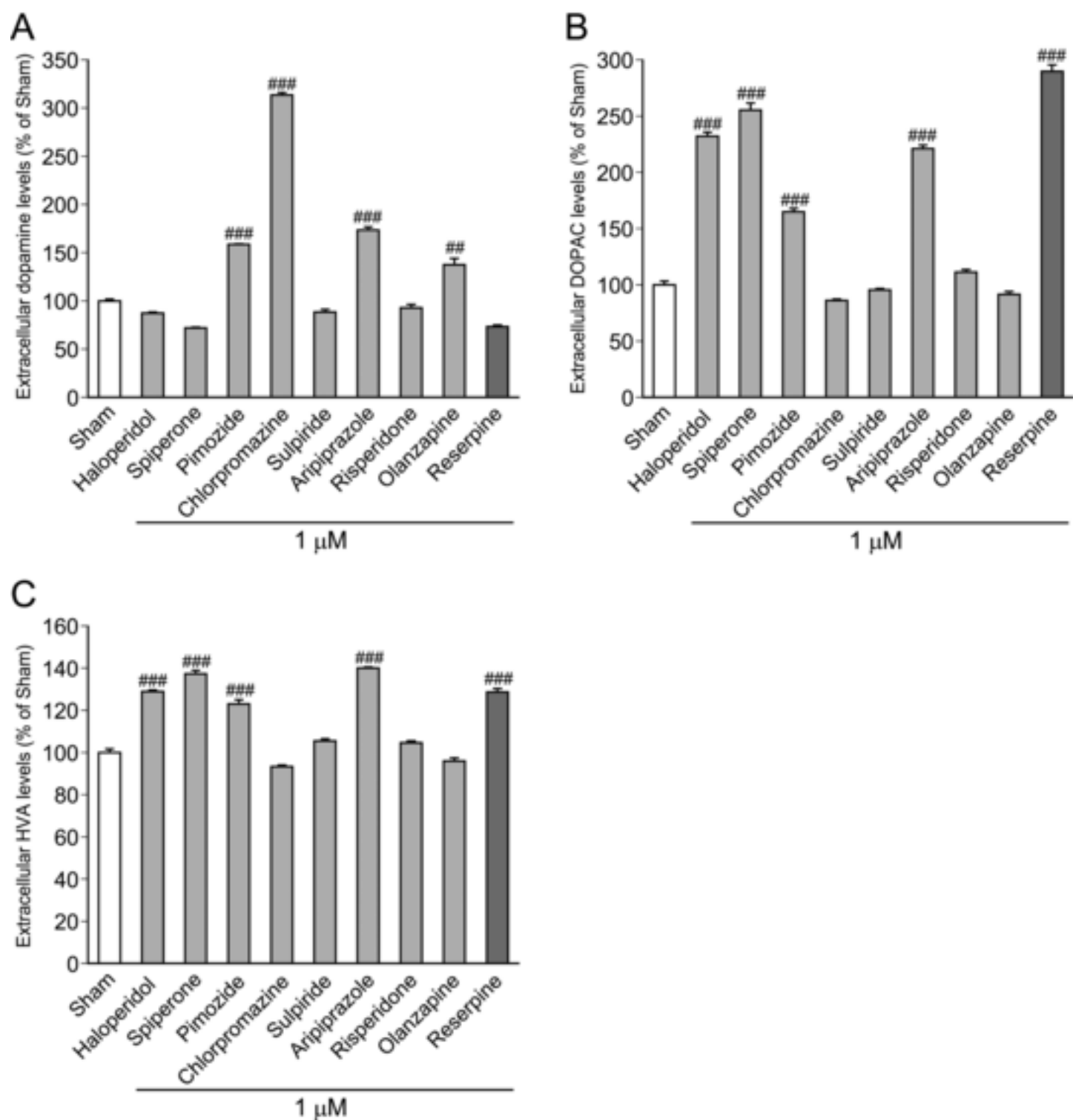


Figure 6 Matsuo et al.

Figure 7
[Click here to download high resolution image](#)

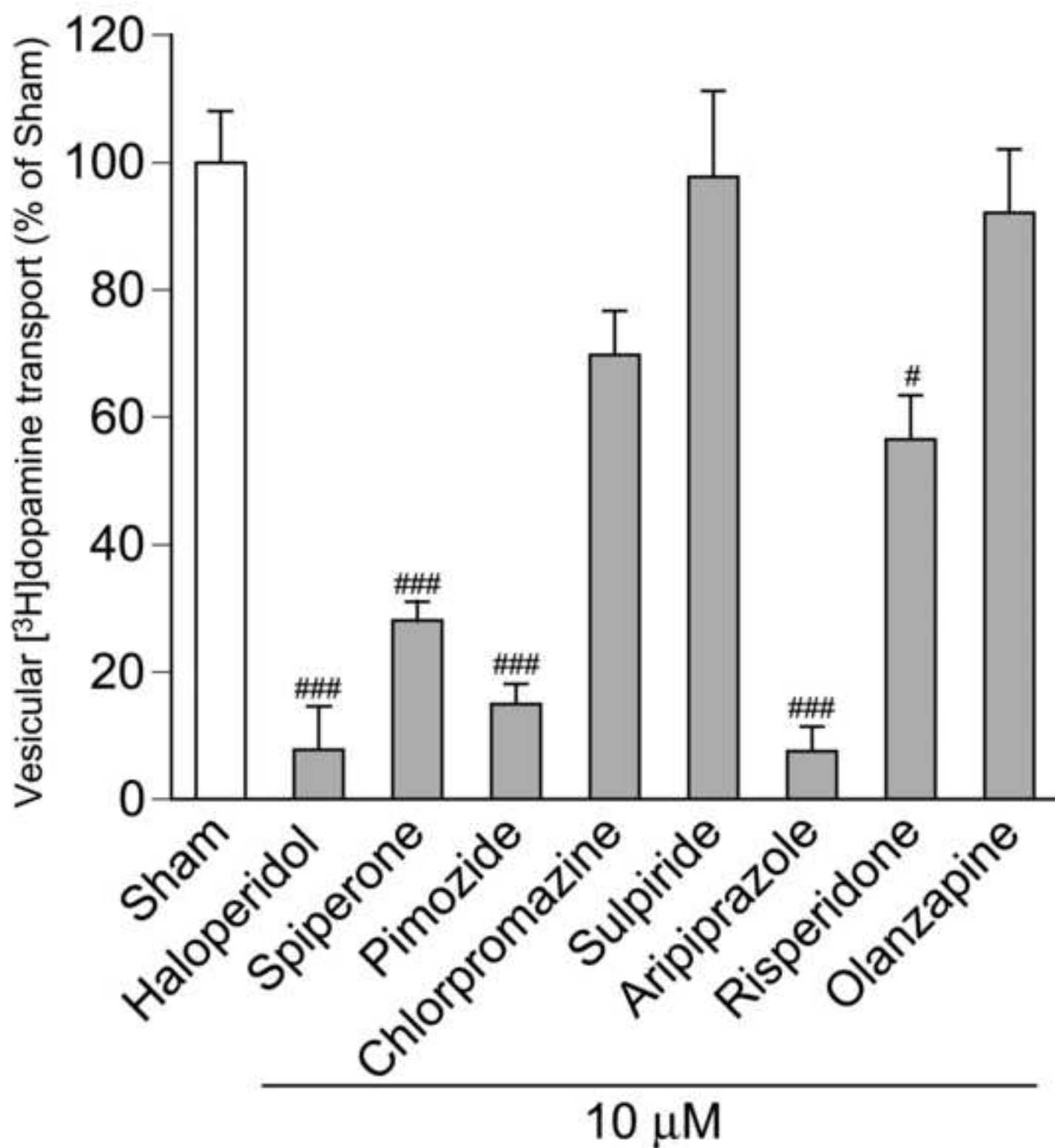


Figure 7 Matsuo et al.