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# Administration of Haloperidol With Biperiden Reduces mRNAs Related to the Ubiquitin-Proteasome System in Mice

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*ABSTRACT* In order to find molecules affected by administration of an antipsychotic drug with an antimuscarinic drug, which is a common prescription used to prevent extrapyramidal adverse effects caused by the antipsychotic drugs, gene expression profiling in the frontal cortex was studied in mice. After 14 days of administration with 2 mg/kg haloperidol, a typical antipsychotic drug, and 2 mg/kg biperiden, a high-affinity antagonist for muscarinic receptors in the brain, ~500 mRNAs related to synaptic function were investigated. The levels of the mRNAs related to the ubiquitin-related systems were significantly reduced after the combined administration. However, the separate administration of either haloperidol or biperiden had little effect on the levels of the mRNAs. This result suggests that coadministration of haloperidol and biperiden specifically affects the ubiquitin-related system. **Synapse 56:175–184, 2005.** 

## INTRODUCTION

Antipsychotic drugs, especially those that possess potent blocking activity for dopaminergic receptors, have extrapyramidal adverse effects. There are six types of adverse effects (Baldessarini and Tarazi, 2001). Acute dystonic reactions, akathisia, Parkinsonism, and neuroleptic malignant syndrome are known to occur shortly after administration of the drugs. On the other hand, perioral tremor and tardive dyskinesia insidiously become obvious only after long periods of drug administration. Among these impairments, acute dystonic reactions and Parkinsonism are effectively treated with antimuscarinic drugs. In order to prevent the adverse effects of the antipsychotic drugs, antimuscarinic compounds are frequently coadministered with antipsychotics.

Although antimuscarinic drugs can suppress the extrapyramidal symptoms, they produce their own adverse effects. Coadministration of an antimuscarinic drug and an antipsychotic drug, or administration of an antipsychotic drug with antimuscarinic activity, have been reported to exacerbate problems with psychological functions, especially memory and attention in schizophrenic patients (Minzenberg et al., 2004; Spohn and Strauss, 1989). After it was first recognized that psychological impairments could occur due to the combined administration of the drugs, many clinical and animal studies were done and they confirmed these psychological adverse effects. Due to the discovery of a beneficial effect provided by atypical antipsychotic drugs in schizophrenic patients with cognitive impairment, there has been an increased prevalence of their use clinically. This has also led to the reevaluation of psychological functions and adverse effects related to drug coadministration. Brébion et al. (2004) reported that the type of antipsychotic drugs (typical vs. atypical) may be less relevant for memory efficiency than their potential antimuscarinic properties. Additionally, McGurk et al. (2004) reported there was an advantageous memory effect for the atypical antipsychotic drug, risperidone, versus that seen for haloperidol. They reported that this

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could be largely explained by the differential antimuscarinic drug administration that is seen in the haloperidol-treated subjects. Although several studies have searched for different molecular mechanisms that occur between typical and atypical antipsychotic drugs, no molecular studies have reported any influence on psychological effects after coadministration of antipsychotic and antimuscarinic drugs. Therefore, we performed a gene expression study to examine the effect of drug coadministration.

Minzenberg et al. (2004) indicated that the antimuscarinic load on antipsychotic drugs is associated with a certain type of cognitive impairment, and they suggested that the dysfunction of the cholinergic systems is related to a specific region that is located in an area that extends from the medial septum to the hippocampus and/or the nucleus basalis of Meynert to the cerebral cortex. The latter cholinergic system is plausible as the source of the responsive region for the psychological impairment seen during drug coadministration, since studies in monkeys as well as in rodents have suggested there is involvement of the nucleus basalis of Meynert in attention (Voytko, 1996), which is impaired during drug coadministration. Moreover, dopamine antagonists ameliorated but did not exaggerate scopolamine-induced impairment of the spatial working memory, for which the hippocampus plays an important role (Kim and Levin, 1996). This opposite effect of an antipsychotic drug on the antimuscarinic drug-induced cognitive impairment is different from the effect of drug coadministration on the psychological functions in schizophrenic patients. Therefore, the frontal cortex but not the hippocampus must be involved in the psychological impairment induced by the drug coadministration.

Dopamine regulates the excitability of the neurons in the frontal cortex and plays an important role in memory and cognition (Cools and Roberts, 2004; Goldman-Rakic, 1998). The cholinergic system in the frontal cortex is also involved in the same psychological functions (Everitt et al., 1997; Sarter and Bruno, 1997; Voytko, 1996). In this study we used haloperidol as the antipsychotic drug, as haloperidol has little antimuscarinic activity but does have potent dopaminergic receptor blocking activities (Baldessarini and Tarazi, 2003) and since it is usually prescribed with an antimuscarinic drug. Biperiden was used as the antimuscarinic drug, as biperiden is prescribed with antipsychotic drugs to prevent extrapyramidal adverse effects (Sadock and Sadock, 2003) and since it also has a high affinity for the CNS (Syvalahti et al., 1988).

Adaptor-tagged competitive PCR (ATAC-PCR) was used in the study. We chose this method because there is only a small deviation within the data that is normally seen. Specific alterations in subgroups of cells in the brain are diluted due to the fact that the brain tissue homogenate is highly heterogenous (Wurmbach et al., 2002). Therefore, a quantification method that has only a small deviation is required in order to be able to document significant alterations when the value of the change is very small. With the ATAC-PCR procedure, the templates from both the control and drug-treated mice are competitively amplified using a universal primer in the same tube, and thus there is only a small deviation noted. The second reason that we used ATAC-PCR is that we measured mRNAs that are not included in ready-made microarrays. Although technical progress has enabled microarray experiments to measure 20,000 mRNAs in a single hybridization, there are still some genes that are yet to be included in the ready-made arrays.

In this study we performed gene expression profiling after the administration of haloperidol and biperiden in order to examine the effect of these drugs on the transcriptosome in the frontal cortex. ATAC-PCR was used to quantify the mRNA levels and proved to be able to document a specific alteration in the levels of a subgroup of mRNAs.

## MATERIALS AND METHODS

### Animals and drugs

Male 9–12-week-old ddY mice (Japan SLC, Hamamatsu, Japan) weighing 40–50 g were used. Animals were housed with free access to standard food in an air-conditioned room under a constant dark-and-light cycle (light on 7:00 AM to 7:00 PM) at a temperature of 22–24°C and 60–70% relative humidity. Pentobarbital (50 mg/kg, i.p.) was used for anesthesia during decapitation. All efforts were made to minimize animal suffering and to reduce the number of animals used. The present experiments were carried out after obtaining permission from the Committee of Animal Experimentation of the Graduate School of Medical and Dental Sciences at Kagoshima University.

Doses of 2 mg/kg haloperidol (Yoshitomi Pharmaceutical, Osaka, Japan) and 2 mg/kg biperiden (Dainippon Pharmaceutical, Osaka, Japan) were used in the study. These drugs were dissolved in 0.9% saline and were injected intraperitoneally in a volume of 0.1 ml/10 g body weight. Haloperidol, biperiden, haloperidol + biperiden, or saline were administered once daily for 14 consecutive days. The 2 mg/kg haloperidol dose was chosen since this dose of haloperidol induces catalepsy in almost all mice (Oka et al., 1979). Also, administration of 2 mg/kg haloperidol has been reported to yield plasma levels in rats that are relevant to that of humans who receive standard doses of haloperidol (Mahadik et al., 1988; Terry et al., 2003). Since biperiden is frequently prescribed at approximately the same dosage as haloperidol in schizophre-

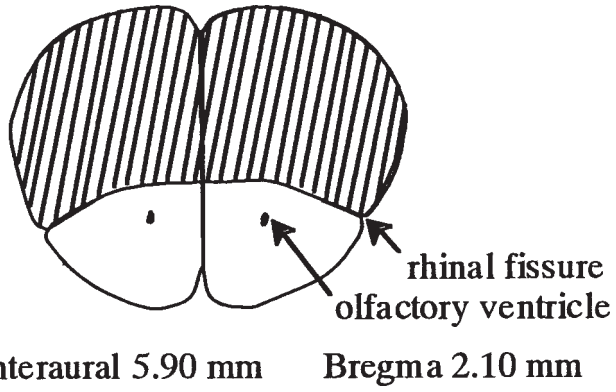


Fig. 1. Brain tissues used are indicated by the oblique strips (Reprinted from "The Mouse Brain," Paxinos et al., (2001), with permission from Elsevier.)

nic patients (Sadock and Sadock 2003), we therefore adopted a 2 mg/kg biperiden dose for this study.

## ATAC-PCR

In the present study the genes to be investigated were selected from the OMIM (Online Mendelian Inheritance in Man) database using the keywords "neurite," "death," and "apoptosis" in order to determine the functions and activities of the neurons. These genes were determined to be appropriate for investigation in the present study. Some genes that were not listed in the OMIM database were added if these genes were related to the selected genes, e.g., synaptotagmins, MAP kinases, cyclin-dependent kinases, etc. The complete list of genes investigated in the present study is available on the internet at http://www.kufm.kagoshima-u.ac.jp/~pharmaco/english/table2.html. The levels of the mRNAs were quantified using ATAC-PCR. ATAC-PCR was essentially performed as described previously (Iwata et al., 2004). Mice were decapitated at 48–53 h after the last drug injection. The rostral part of the frontal cortex (Fig. 1) was immediately removed and total RNA was then extracted using the RNAgents Total RNA Isolation System (Promega, Madison, WI). cDNA was synthesized using 15-pmol biotinylated oligo-dT18 primer, and cDNA was then digested by either Mbo I or Hha I. Digested cDNA was ligated either with a short or a long adaptor. These adaptors shared the same primer sequence, but had different numbers of spacer nucleotides between the primer and cohesive end (1 bp in the short adaptor and 4 bp in the long adaptor) so that we could discriminate by DNA sequencing between the cDNA derived from controls and that derived from drug-treated mice. The sequences of the adaptors for the Mbo I-digested cDNA were as follows:

- Short adaptor: 5′-GTACATATTGTCGTTAGAACG-CG-3′ 3′-CATGTATAACAGCAATCTTGCGCCTAG-5′ Long adaptor: 5′-GTACATATTGTCGTTAGAACGC-GACT-3′ 3′-CATGTATAACAGCAATCTTGCGCTGAC TAG-5′
- Short adaptor: 5′-GTACATATTGTCGTTAGAACGCG-3′ 3′-CATGTATAACAGCAATCTTGCGCCGCG-5′ Long adaptor: 5′-GTACATATTGTCGTTAGAACGCGACT-3′ 3′-CATGTATAACAGCAATCTTGCGCTGACGCG-5′

Spacer nucleotides are underlined.

Saline-injected control cDNA, which was bound to one of two adaptors, and drug-injected cDNA, which was bound to the other adaptor, were mixed in the same tube, and then streptavidin-coated paramagnetic beads (M-280, Dynal, Oslo, Norway) were added to bind the 3′ end of the cDNA. PCR was performed with the beads using FAM-labeled universal primer, which is the complement to both adaptors (5′-GTACATATTGTCGTTAGAACGC-3′), and a gene-specific primer. PCR products were electrophoresed using a DNA sequencer. The lengths of the PCR products and their fluorescence intensity were measured using GeneScan analysis software v. 3.7 (Applied Biosystems, Foster City, CA). The fluorescence intensity units were converted into percentage values in which averages of fluorescence intensity units from the corresponding saline-treated controls were set at 1. Significant changes in the level of the mRNAs were determined after normalizing the levels of actin mRNA or GAPDH mRNA, with the values being set to 1. Statistical analysis was performed using ANOVA with Fisher PLSD post-hoc tests.

## RESULTS

Out of ~500 mRNAs listed as genes related to neurite, survival, and death, ~100 mRNAs could be amplified by ATAC-PCR in the frontal cortex. Of these, 51 mRNAs could be detected in more than three out of six mice in all of the experimental groups, i.e., saline-treated mice, haloperidol-treated mice, biperiden-treated mice, and (haloperidol + biperiden)-treated mice. These genes were used for the statistical analysis.

After coadministration of haloperidol and biperiden, the level of the mRNAs for the ubiquitin-conjugating enzyme E1 (UBE2E1), ubiquitin C-terminal hydrolase L5 (UCHL5), and UCHX4 decreased in comparison with the saline-treated control and haloperidol-treated groups. The level of the mRNAs for the 14-3-3ζ, activating transcription factor 4 (ATF4), high mobility group box 1 (HMGB1), MAP3K4, UBE2E1, UCHL5, and UCHX4 decreased in comparison to the haloperidol-treated group (Fig. 2). After haloperidol administration, only the HMGB1 mRNA level showed a significant increase, with all other mRNA levels exhibiting no significant change. After biperiden adminis-

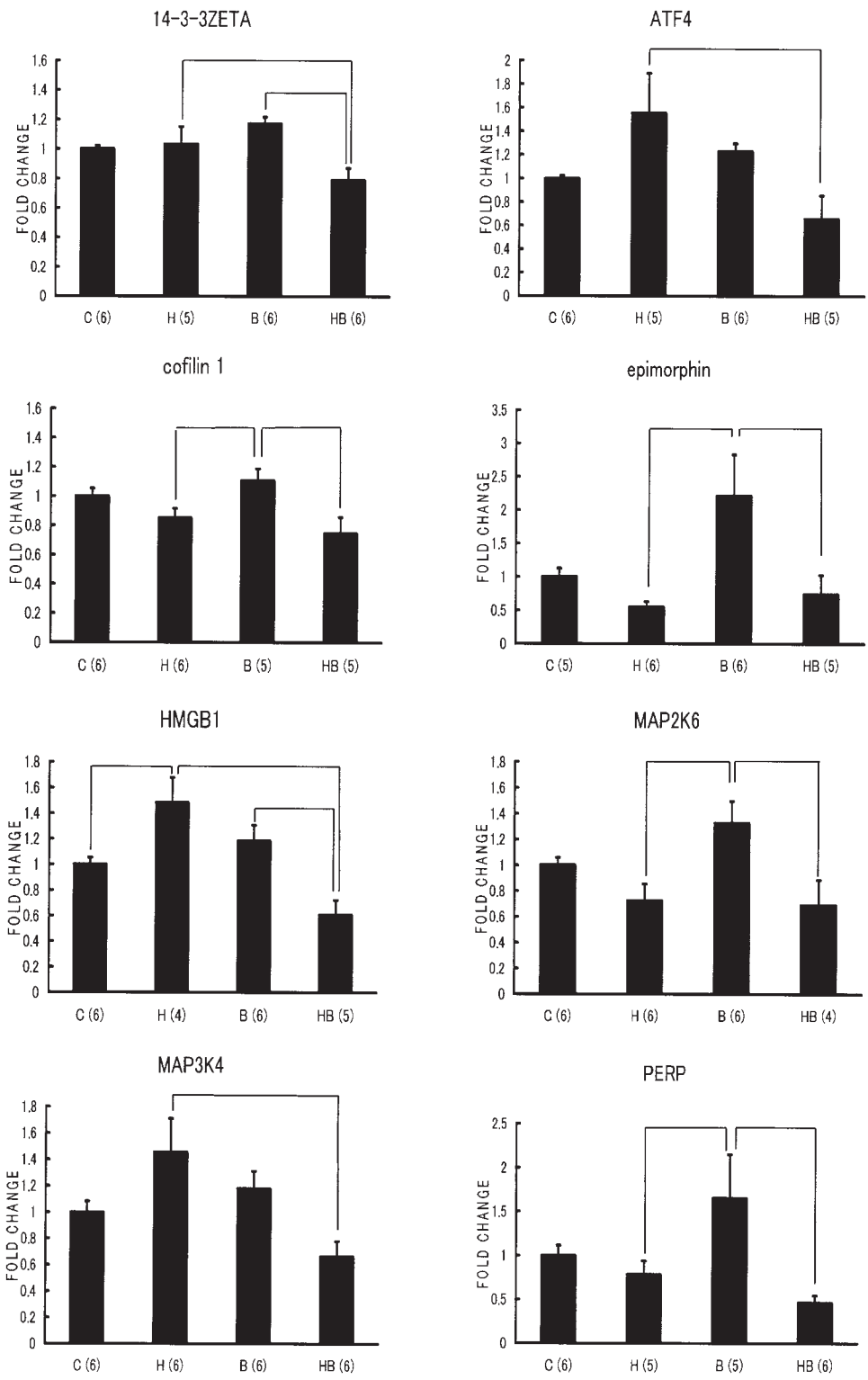


Fig. 2. Alterations seen in the level of the mRNAs after a 14-day administration of haloperidol, biperiden, or coadministration of haloperidol and biperiden. Only genes that showed significant alterations in the level of their expression are shown ($P < 0.05$, ANOVA). Fluorescent intensity units for each gene are shown relative to the average of the fluorescent intensity unit in the control, which was set at 1. Vertical bars express standard error. C, saline-administered mice; H, haloperidol-administered mice; B, biperiden-administered mice; HB, (haloperidol + biperiden)-administered mice. Significant alterations ($P < 0.05$; Fisher PLSD) between the groups are connected by a line. The numbers in parentheses indicate the number of mice in which the PCR product was successfully obtained. Six mice were used in each group. ATF4, activating transcription factor 4; HMBG1, high mobility group box 1; PERP, p53 apoptosis effector related to PMP-22. UBE2B, ubiquitin-conjugating enzyme B; UCHL5, ubiquitin C-terminal hydrolase L5.

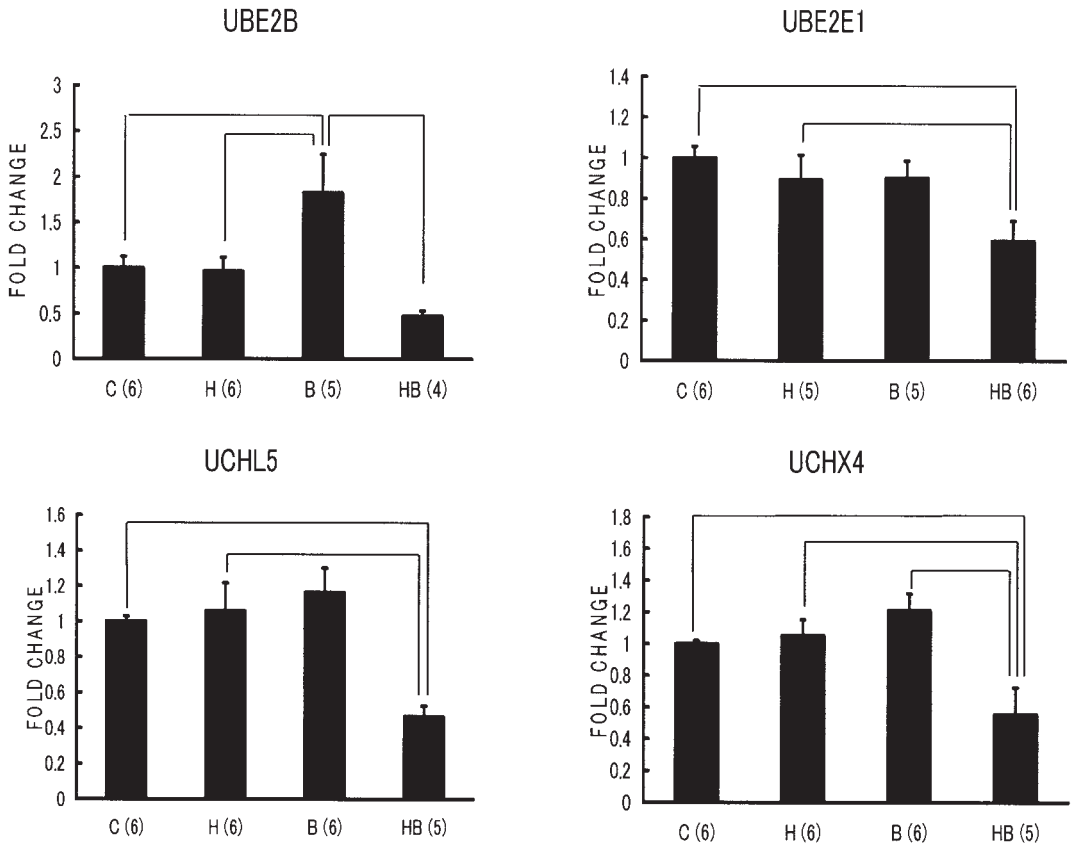


Figure 2 (Continued.)

tration, only the UBE2B mRNA level increased, with no changes noted in the levels for any of the other mRNAs.

The average levels of the ubiquitin-proteasome system-related genes, i.e., UBE2B, UBE2D1, UBE2E1, UBE2E3, UBP, UCHL1, UCHL5, UCHX4, and VCP observed after the coadministration of haloperidol and biperiden were significantly lower than those seen for the mRNAs of the nonubiquitin-proteasome system-related genes ($P < 0.0001$, Mann-Whitney U-test) (Fig. 3C). There were no significant differences between the mRNA levels in these two groups after either haloperidol or biperiden administrations (Fig. 3A,B).

The pro-apoptotic mRNAs that were not detected in the saline-treated mice were also not amplified in either the haloperidol-treated mice or in the (haloperidol + biperiden)-treated mice. These mRNAs included the apoptosis-associated tyrosine kinase (AATYK), acinus, Bim, caspase 1, caspase 2, caspase 3, caspase 6, caspase 7, caspase 8, caspase 9, caspase 11, caspase 12, caspase 14, death domain-associated protein (DAXX), DR5, ectodermal neural cortex with BTB-like domain 1 (ENC1), FAS, lost on transformation protein 1 (LOT1), Noxa, and the p53-upregulated modulator of apoptosis (PUMA).

## DISCUSSION

Coadministration of haloperidol and biperiden reduced the level of several mRNAs, in contrast to only slight alterations that were seen for the mRNA levels after the separate administrations of either haloperidol or biperiden. UBE2E1, UCHL5, and UCHX4 showed the most significant reductions in their expression levels. UBE2E1 is a mouse homolog of yeast UBC4/5, which is one of the ubiquitin-conjugating enzymes. UCHL5 and UCHX4 are ubiquitin-recycling enzymes. We found that not only the levels of UBE2E1, UCHL5, and UCHX4, but also the levels of most of the ubiquitin-related mRNAs, decreased after the drug coadministration (Fig. 3C). This result suggests a significant relationship between the drug coadministration and the ubiquitin-related systems.

Although the mechanism of the decrease in the ubiquitin-related mRNAs is unclear, it can be hypothesized that there is a feedback system. Chronic administration of haloperidol upregulates the dopaminergic receptors in the frontal cortex (MacLennan et al., 1988). Similarly, chronic biperiden administration must upregulate muscarinic receptors in the frontal cortex, as chronic administration of scopolamine has been shown to upregulate muscarinic receptors in the frontal cortex (Pietrzak et al., 1989; Pilch

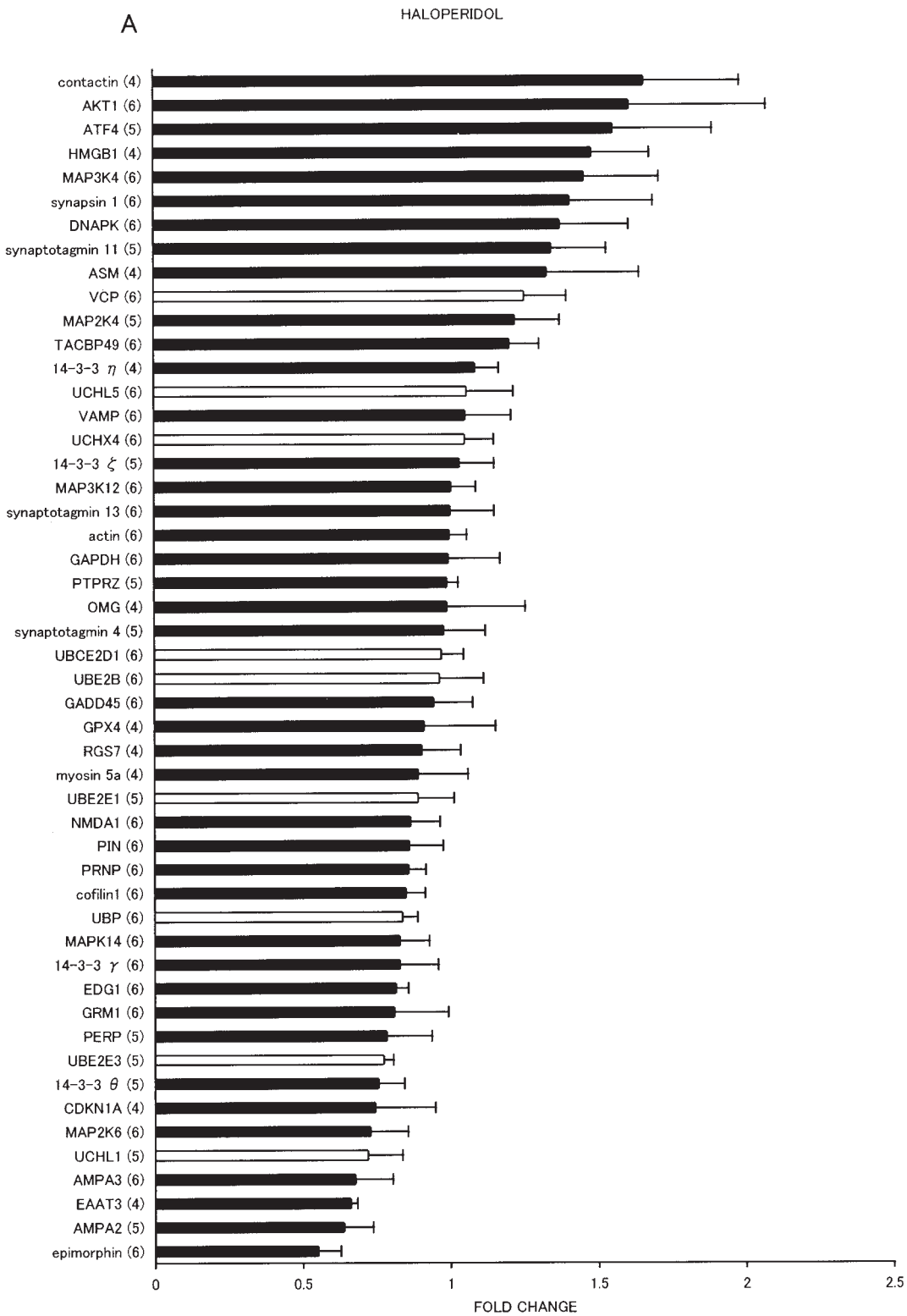


Fig. 3. The level of all mRNAs quantified after a 14-day administration of haloperidol (**A**), biperiden (**B**), or haloperidol + biperiden (**C**). The numbers in parentheses indicate the number of mice in which the PCR product was successfully obtained out of six mice. Open columns indicate the ubiquitin-proteasome system-related genes. Closed columns indicate the rest of the genes. A Mann-Whitney U-test was used to calculate significant differences in the average levels of gene expression between the ubiquitin-related genes and the rest of the genes. ASM, acid sphingomyelinase; ATF4, activating transcription factor 4; CDKN1A, cyclin-dependent kinase inhibitor 1A; DNAPK, DNA-dependent protein kinase; EDG1, endothelial differentiation gene 1; EAAT3, excitatory amino acid transporter 3; GRM1, glutamate receptor, metabolic 1; GPX4, glutathione peroxidase X4; GADD45, growth arrest- and DNA-inducible gene 45; GAS5, growth arrest-specific 5; HMBG1, high mobility group box 1; OMG, oligodendrocyte-myelin glycoprotein; PERP, p53 apoptosis effector related to PMP-22. PIN, protein inhibitor of neuronal NOS; PRNP, prion protein; PTPRZ1, protein-tyrosine phosphatase, receptor type, zeta 1; RGS7, regulator of G protein signaling 7; TACBP49, taipoxin-associated calcium binding protein 49; UBE2B, ubiquitin-conjugating enzyme B; UBP, ubiquitin-specific processing protease; UCHL5, ubiquitin C-terminal hydrolase L5; VCP, valosin-containing protein; VAMP, vesicle-associated membrane protein.

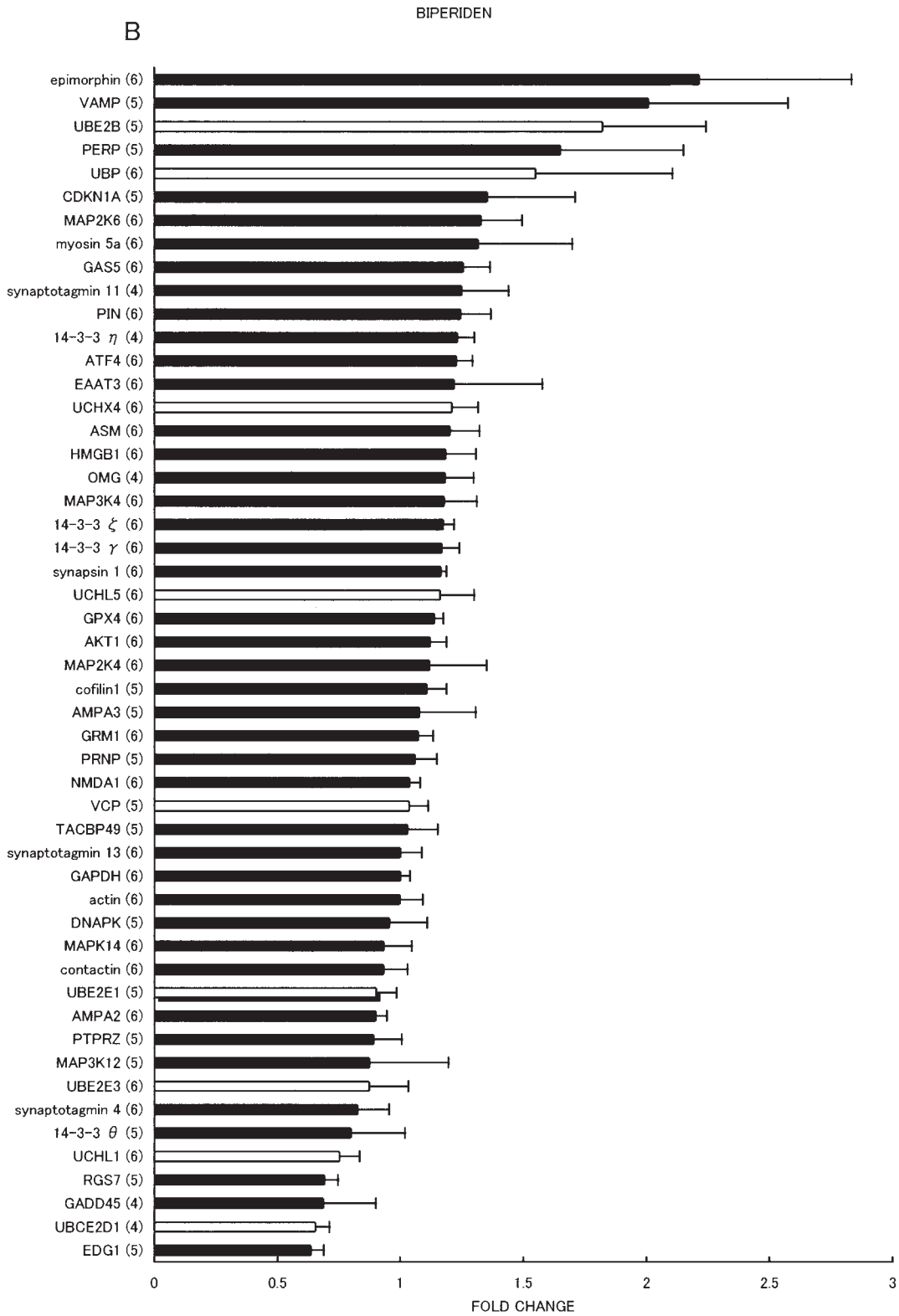


Figure 3 (Continued.)

and Müller, 1988). Upregulation of receptors by chronic antagonist administration is due to an inhibition of agonist-induced receptor sequestration (Tsao et al., 2001). After binding with agonists, receptors are sequestered from the surface of the synaptic membrane by endocytosis. After a long period of inhibition of agonist-induced endocytosis due to chronic administration of antagonists, receptors remain on

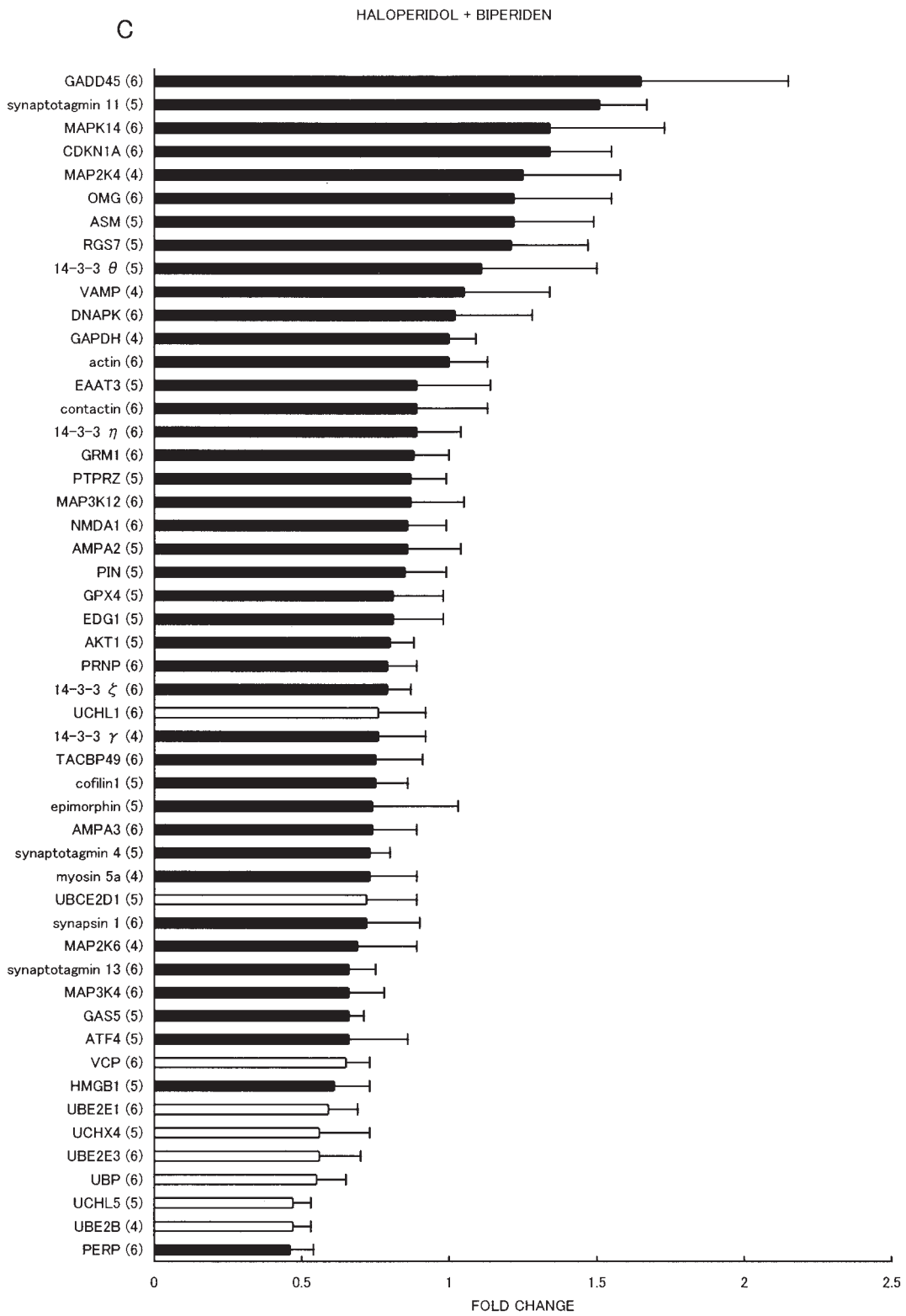


Figure 3 (Continued.)

the surface of the synaptic membrane. Although phosphorylation of receptors has been considered the main mechanism for agonist-induced endocytosis, it has lately been recognized that ubiquitination plays an important role in the receptor sequestration (Hegde and DiAntonio, 2002). Therefore, levels of

mRNAs that are related to the ubiquitin-dependent receptor sequestration could be subject to feedback-regulation after a long-term haloperidol and biperiden administration.

Occurrence of the same feedback mechanism for the regulation of mRNA levels is possible in both the presynaptic region as well as the postsynaptic site. Chronic haloperidol administration has been observed to attenuate high $K^+$-induced dopamine release in the frontal cortex (See et al., 1995; Yamamoto and Cooperman, 1994), and chronic biperiden administration must also inhibit acetylcholine release in the frontal cortex. Presynaptic molecules required for neurotransmitter release are digested by the ubiquitin-proteasome system in the nerve terminals and the inhibition of the ubiquitin-proteasome system enhances neurotransmission (DiAntonio and Hicke, 2004; Speese et al., 2003). Therefore, mRNAs related to this system can be regulated by the continuous inhibition of neurotransmitter release through chronic drug coadministration. Regulation of mRNA levels related to the neurotransmitter system after long-term agonist administration has been reported in the dopaminergic system (Iwata et al., 2000).

Most of the ubiquitin-related mRNAs examined in this study decreased. However, not all mRNAs related to ubiquitin in our study play a part in the ubiquitin-related neurotransmission, as functions of the ubiquitin-dependent systems are very diverse. Therefore, it can be hypothesized that ubiquitin-related systems other than ubiquitin-related neurotransmission could be involved in the feedback regulation that is seen during drug coadministration. The possibility exists that the ubiquitin-dependent protein digestion system could be affected by drug coadministration.

It has been established that ubiquitin-dependent mechanisms are involved in memory formation. This was first suggested for the long-term potentiation (LTP) that is seen in *Aplysia* (Hegde et al., 1997). In mammals, the ubiquitin-proteasome cascade has also been proven to be crucial for the establishment of long-term memory (Jiang et al., 1998; Lopez-Salon et al., 2001). Therefore, drug coadministration may impair memory by way of hypofunction of the ubiquitin-dependent systems, even though alterations in the levels of mRNAs do not translate into exact parallel changes in protein levels. Further proteasome studies on ubiquitin-related proteins are needed to demonstrate this hypothesis.

A mechanism that may explain why coadministration but not separate drug administration reduced the levels of mRNAs may involve the dopamine-acetylcholine interaction. Long-term haloperidol administration has been reported to reduce the level of the immunoreactivity of choline acetyltransferase in the rat frontal cortex (Angelucci et al., 2000; Terry et al., 2003). It is possible that postsynaptic muscarinic receptors are upregulated by this drug-induced denervation (Crook et al., 2001). Therefore, concomitant administration of biperiden will prevent the physiological adaptation of the muscarinic receptor-induced signal transduction. Overall this means that drug coadministration affects both the presynaptic and postsynaptic cholinergic systems. Thus, levels of ubiquitin-related mRNAs may only decrease when both pre- and postsynaptic sites are affected.

It is uncertain whether alterations in the levels of mRNAs are specific to the frontal cortex, as no similar experiments have been performed in other regions of the brain. Since both haloperidol-induced extrapyramidal symptoms and the ameliorating effect of the extrapyramidal symptoms by antimuscarinic drugs occur via the striatum, mRNA levels must also change in the striatum. Additionally, there have been many studies that reported alterations in the level of mRNAs in the striatum after long-term haloperidol administration. Although ATAC-PCR provides distinct advantages, using a single-capillary sequencer to quantify 500 mRNAs from plural brain regions in several animals by ATAC-PCR is very laborious. Therefore, we were only able to perform the gene expression study in the frontal cortex.

In conclusion, the present results indicate that the coadministration of haloperidol and biperiden reduces the level of ubiquitin-related mRNAs. This alteration is specific for drug coadministration, as the effect is not seen during the separate administration of either haloperidol or biperiden. We believe that there could possibly be a specific mechanism that affects all ubiquitin-related mRNAs. Further proteasomal studies are needed to elucidate whether these mRNA changes cause psychological impairment or if they are simply an adaptive change related to the chronic drug coadministration.

## ACKNOWLEDGMENT

The authors thank Ms. K. Godo for help with the gene list table, which can be found at: http://www.kufm.kagoshima-u.ac.jp/~pharmaco/english/table2.html.

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