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Brain regions and monoaminergic neurotransmitters that are involved in mouse ambulatory activity promoted by bupropion

Toyoshi Umezu *, Yasuyuki Shibata

Biological Imaging and Analysis Section, Center for Environmental Measurement and Analysis, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan

ABSTRACT

Bupropion (BUP), a substituted phenyl-ethylamine, has been utilized for the treatment of depression and for smoking cessation, however, one concern is that BUP may increase a risk of psychosis similar to other substituted phenyl-ethylamine amphetamine (AMPH) and methamphetamine (MetAMPH). BUP promotes ambulation in mice and causes behavioral sensitization on the ambulation-promoting effect when repeatedly administered as well as AMPH and MetAMPH. The present study aimed to elucidate brain regions and monoaminergic neurotransmitters that are involved in the ambulation-promoting effect of BUP. c-Fos-like immunoreactivity (c-Fos-IR) mapping in brain in combination with measuring ambulatory activity was conducted to determine brain region(s) that is involved in the ambulatory effect of BUP. Three kinds of statistical analyses for c-Fos-IR in 24 brain regions consistently showed that c-Fos-IR in the Caudate putamen (CPu) is positively correlated with the ambulatory response to BUP. In addition, multiple regression analysis indicated that the ambulatory response is a function of c-Fos-IR not only in the CPu but also in the lateral septum nucleus (LS), median raphe nucleus (MnR), lateral globus pallidus (LGP), medial globus pallidus (MGP), locus coeruleus (LC) and ventral hypothalamic nucleus (VMH). Effects of BUP on monoaminergic neurotransmitters in the CPu were examined using *in vivo* microdialysis method, as the pharmacological experiments indicated that monoaminergic neurotransmitters, dopamine (DA) in particular, mediate the ambulatory response to BUP. Response of DA in the CPu to BUP was parallel to the ambulatory response, showing that DA in the CPu is involved in the ambulatory response to BUP. The present study also suggests that other brain regions such as the LC, the origin nucleus of norepinephrine (NE) neurons, and another neurotransmitter NE may also play some roles for the ambulatory response to BUP, however, further studies are needed to elucidate the roles.

1. Introduction

Bupropion (BUP), a substituted phenyl-ethylamine, was found to possess therapeutic benefits as an atypical antidepressant medication [18], smoking-cessation medication [18] and drug abuse-cessation medication [10,14,46]. Clinical use of BUP for the treatment of depression and for smoking-cessation was approved by the Food and Drug Administration (FDA) in the United States. Daily dose of 450 mg is recommended for the treatment of depression. The original immediate release formulation of BUP is dosed three times daily, followed by introduction of the sustained-release formulation that is dosed twice daily and the extended-release formulation that is dosed once daily [29].

Though BUP has been extensively utilized for the treatment of depression and for smoking cessation, one concern is that BUP may increase a risk of psychosis [29]. Several studies report that BUP may cause or worsen psychosis [6,21,25,27,62]. BUP inhibits the dopamine (DA) transporter (DAT) and the norepinephrine (NE) transporter (NET), and is an antagonist at the neuronal nicotinic acetylcholine receptors (nAChRs) [48,50]. The IC_{50} of BUP for inhibiting DA uptake is higher than the IC_{50} values for inhibiting NE uptake and nAChRs function [16]. This pharmacological property also suggests that BUP can have the potential for precipitating psychosis. However, whether or not BUP increase a risk of psychosis and neuronal mechanisms underlying the potential of BUP to increase the risk have not been elucidated sufficiently [29].

BUP promotes ambulatory activity in mice [54,55,59]. Repeated administration of BUP to the same mouse augments the ambulation-promoting effect (behavioral sensitization) [58]. These ambulatory effects of BUP are similar to those of amphetamine

(AMPH) and methamphetamine (MetAMPH) [22,30], other substituted phenyl-ethylamine, that have the potential to produce psychosis-like mental disorder (amphetamine psychosis) [26]. Mechanism underlying the locomotion-promoting effect of AMPH in mice is elucidated well [7,24,36,51,64], whereas mechanisms underlying the ambulation-promoting effect of BUP have poorly been elucidated.

The present study aimed to elucidate brain region(s) and neurotransmitter(s) that are involved in the ambulation-promoting effect of BUP.

Mapping c-Fos-like immunoreactivity (c-Fos-IR), which indicates neuronal activation, is useful for determining brain regions that are activated by CNS acting drugs and involved in specific behaviors [43,66]. BUP increases c-Fos-IR in various rat brain regions [8], whereas effects of BUP on c-Fos-IR in mouse brain have not been studied. The present study conducted c-Fos-IR mapping in combination with measuring ambulatory activity to determine mouse brain region(s) that is involved in the ambulatory response to BUP.

BUP inhibits dopamine (DA) transporter (DAT) and norepinephrine (NE) transporter (NET) and enhances the extracellular DA and NE levels in rat brain [11,33]. On the other hand, effects of BUP on monoaminergic neurotransmitters in mouse brain have not been elucidated well. The present study examined whether or not monoaminergic neurotransmitters mediate the ambulatory response to BUP using drugs that modulate monoaminergic neurotransmission. Drugs used were α-methyl-*p*-tyrosine (AMPT), reserpine (RES), chlorpromazine (CPZ), fluphenazine (FLU), SCH12679 (SCH), spiperone (SPI), haloperidol (HAL) and pimozide (PIM). AMPT and RES deplete monoaminergic neurotransmitters in brain [17,41,61]. CPZ, FLU, SCH, SPI, HAL and PIM antagonize DA receptors (DARs) and their affinities for DARs are much higher than those for NE receptors [13,47]. Then, the present study examined effects of BUP on monoaminergic neurotransmitters in brain region(s) that was identified by the c-Fos-IR study using *in vivo* microdialysis in order to elucidate relationships between responses to BUP of monoaminergic neurotransmitters in the brain region(s) and of the ambulatory activity.

2. Materials and methods

2.1. Subjects

Male ICR strain mice (Clea Japan, Tokyo, Japan) aged 7–15 weeks and weighing 35–45 g at the start of experiments were used. Mice were housed in aluminum cages (three mice/cage) with a stainless-steel mesh top and paper bedding. Commercial solid food (Clea Japan) and tap water were provided ad libitum. The cages were placed in a room artificially illuminated by fluorescent lamps on a 12-h light:12-h dark schedule (light period: 07:00–19:00) and a room temperature of $25 \pm 1\,°C$. All experiments were conducted during the light phase.

All experiments were performed in accordance with the guidelines of the Ethics Committee for Experimental Animals of the National Institute for Environmental Studies, Japan.

2.2. Drugs

Bupropion HCl (BUP), chlorpromazine HCl (CPZ), fluphenazine 2HCl (FLU), SCH12679 ((R)-(−)-Phenyl−2,3,4,5-tetrahydro-1*H*-7,8-dimethoxy-3-benzazepine) maleate (SCH) and spiperone (SPI) (Sigma-Aldrich, Tokyo, Japan) were prepared in 0.9% NaCl (Nacalai Tesque, Kyoto, Japan) solution (saline). Haloperidol (HAL) (Sigma-Aldrich) was prepared in 0.1% acetic acid (Nacalai Tesque) solution. Pimozide (PIM) (Sigma-Aldrich), α-methyl-*p*-tyrosine (AMPT) (ICN

Biomedicals, Solon, OH, USA), and reserpine (RES) (Sigma-Aldrich) were mixed with a small amount of polyoxyethylene sorbitan monooleate (Tween 80) (Nacalai Tesque) and diluted in saline. Doses of BUP, CPZ, FLU, and SCH were expressed as the salt weights.

AMPT was intraperitoneally administered to mice, and other drugs were subcutaneously administered. The administration volume was 1 ml/100 g body weight regardless of the type of drug and dosage.

2.3. Measurement of mouse ambulatory activity

Ambulatory activity was measured using an ambulometer (SAM-10; O'Hara and Co., Tokyo, Japan) [55–59,54,52,53,57] (Appendix A). Each activity cage (20 cm in diameter) is supported by a fulcrum in the center of the bottom; the fulcrum tilts according to movement of the mouse in the activity cage. The tilting movement of the activity cage activates three micro-switches that surround the cage. The number of activations of micro-switches during a set time is recorded, and the result is printed out.

After adapting mice to the activity cages for 30 min, saline, 5 mg/kg or 10 mg/kg of BUP was administered to the mice, followed by measuring the ambulatory activity for 60 min. Combined administration of saline or 50 mg/kg AMPT plus saline or 2, 4, or 8 mg/kg RES was performed on mice at the same time one day before measuring the ambulatory response to 10 mg/kg BUP. Drugs that preferentially antagonize DARs were administered after 30 min of adaptation, 10 min later followed by measuring the ambulatory response to 10 mg/kg BUP.

2.4. Immunocytochemistry for c-Fos

2.4.1. Preparation of brain samples

The following procedures were performed in three separate experiments on mice given saline (n = 8) or BUP (n = 11). We obtained seven brain samples from mice given saline and 10 brain samples from mice given BUP. One brain sample from each of the animal groups was excluded because of unsuccessful transcardial perfusion of fixation.

Individual mice were placed in activity cages, and 30 min later, saline was administered, followed by measurement of ambulatory activity for 60 min. After the measurement, they were returned to their home cages. This procedure was repeated every day for 3 days to reduce stress to the mice (acclimation procedure), because stress induces c-Fos-IR in various brain regions [3,23]. On the 4th day, individual mice were placed in activity cages. After 30 min of adaptation, saline or 10 mg/kg BUP was administered, and ambulatory activity was measured for 60 min. Immediately after the end of the ambulatory measurements, the mice were deeply anesthetized with pentobarbital (Nembutal®, Sumitomo Dainippon Pharma Co., Ltd., Osaka, Japan) and perfused transcardially with saline containing heparin (Wako Pure Chemical Ind. Ltd., Osaka, Japan) followed by Speh's fixative (4% paraformaldehyde, 0.2% saturated picric acid, and 0.05% glutaraldehyde in 0.1 M phosphate buffer pH 7.4), which is a modification of Zamboni's fixative [65]. Brains were removed and post-fixed in the same fixative overnight at 4 °C. They were soaked in 0.1 M phosphate buffer (pH 7.4) containing 25% sucrose for cryoprotection until they had completely sunk. Brains were individually frozen using methyl butane cooled by dry ice and stored at −80 °C.

2.4.2. Immunocytochemistry for c-Fos in brain samples

Coronal sections of brains from the olfactory bulb to the midbrain were cut at a thickness of 50 μm using a cryostat. Immunocytochemistry was performed on free-floating sections in four separate batches. Each batch included samples derived from mice administered either saline or BUP to ensure that the extent of

staining was balanced between the two groups. After washing with Tris-buffered saline (TBS, pH 7.4) (Nacalai Tesque), sections were incubated for 3 days at 4 °C in primary antibody to c-Fos (F7799; Anti-c-Fos rabbit IgG, Sigma-Aldrich; 1:5000) in antibody diluent (TBS containing 0.25% λ-carrageenan, 1% bovine serum albumin, and 0.3% Triton X-100 (all from Sigma-Aldrich)) with 0.1% sodium azide (Nacalai Tesque). Sections were then washed in TBS and incubated in biotinylated secondary goat anti-rabbit IgG (Vector Labs, Burlingame, CA, USA; 1:500) in antibody diluent for 60 min at room temperature. After washing with TBS, sections were incubated with ABC complex (ABC Elite kit, Vector Labs; 1:750) in antibody diluent for 90 min. Staining was visualized by reaction with H_2O_2 and diaminobenzidine (Sigma-Aldrich). The reaction was stopped by washing sections in TBS. Sections were mounted onto subbed slides, allowed to air dry, dehydrated, and coverslipped using Permount (Sigma-Aldrich).

2.4.3. Quantification of c-Fos-IR

Microscopic images of stained sections were captured via a camera (DFC490, Leica, Germany) interfaced with a personal computer. Captured images at $100 \times$ magnification were printed in color for quantification of c-Fos-IR (Appendix B).

Twenty four mouse brain regions were selected by referring to the previous study that examined the effects of BUP on c-Fos expression in various rat brain regions [8], and c-Fos-IR in the brain regions were quantified. The numbers of c-Fos-IR nuclei in areas 0.11-0.8 mm^2 in the 24 brain regions were manually counted. The sizes of the examined area were identical throughout each brain region except the dentate gyrus (DG). Because the morphology of the DG was quite different among each brain, the sizes of the areas in the DG ranged from 0.11 to 0.27 mm^2. The other brain regions examined were the anterior olfactory nucleus (AO), orbital cortex (medial, ventral) (MO, VO), primary motor cortex (M1), medial prefrontal cortex (mPFC), lateral septum nucleus (LS), claustrum (Cl), nucleus accumbens (NAcb), olfactory tubercle (Tu), ventral pallidum (VP), anterior paraventricular thalamic nucleus (PVA), lateral globus pallidus (LGP), caudate putamen (CPu), central amygdaloid nucleus (CeA), medial globus pallidus (MGP), lateral hypothalamic nucleus (LH), ventral hypothalamic nucleus (VMH), subthalamic nucleus (STh), ventral tegmental area (VTA), substantia nigra pars compacta (SNC), substantia nigra pars reticulate (SNR), dorsal raphe nucleus (DR), median raphe nucleus (MnR), and locus coeruleus (LC) (Appendix C). The brain regions were identified according to the mouse brain atlas [42].

Counting of c-Fos-IR nuclei in the SNR, CPu, and CeA was performed in duplicate, and their correlations were examined in order to confirm the reliability of our c-Fos-IR quantification. Correlation coefficients were 0.9795, 0.8481, and 0.9606 for the CPu, CeA, and SNR, respectively (Appendix D), showing that the quantification of c-Fos-IR was reliable.

2.5. Measurement of brain monoaminergic neurotransmitters and their metabolites using in vivo microdialysis

Mice were anesthetized using 50 mg/kg pentobarbital (Nembutal®) and fixed in a stereotaxic apparatus equipped with a mouse adapter (David Kopf, CA, USA). A dialysis probe (D-I-6-02, cut-off 50,000 Da, Eicom, Kyoto) was stereotaxically implanted into the brain region determined to be involved in the ambulatory response to BUP by the c-Fos study according to the mouse brain atlas [42] and fixed with dental cement. After surgery, the animals were individually housed in cages and allowed to recover for 2–3 days.

Two to three days after surgery, online measurement of the extracellular monoaminergic neurotransmitters, DA, NE, and 5-HT, and their metabolites, (3,4-dihydroxy-phenylacetic acid (DOPAC),

homovanillic acid (HVA), 3-methoxy-4-hydroxy-phenyl-ethylene glycol (MHPG), and 5-hydroxyindolacetic acid (5-HIAA)) was performed using in vivo microdialysis coupled with high-performance liquid chromatography (HPLC). The probe-implanted mouse was placed in a cage for the microdialysis experiment and allowed to move freely in the cage. Food and water were available ad libitum throughout the microdialysis measurement. Ringer's solution (147 mM Na$^+$, 4 mM K$^+$, and 2.3 mM Ca$^+$, 155.6 mM Cl$^-$) was perfused at a rate of 2.0 μl/min through the probe using a syringe pump (ESP-64, Eicom). Each dialysate sample was collected for 25 min using an auto injector (EAS-2, Eicom) that automatically injected the dialysate sample into HPLC immediately after the end of each 25-min collection. After at least four dialysate samples were collected to establish baseline levels, saline or 10 mg/kg BUP was administered to the mouse, followed by continuous collection of six dialysate samples. The lag time from the probe tip to the auto injector was considered to determine the time point for the administration. After the end of the microdialysis measurement, eosin solution was perfused through the probe, the mouse was deeply anesthetized using pentobarbital (Nembutal®), and the brain was removed and fixed using 10% formaldehyde neutral buffer solution (Nacalai Tesque). The placement of the probe was verified histologically (Appendix E).

Monoaminergic neurotransmitters and their metabolites in dialysate samples were analyzed using HPLC (HTEC-500, Eicom). Each sample was analyzed for 25 min using HPLC during which all analytes of interest appeared in a chromatogram. Before starting the microdialysis experiment for each mouse, HPLC was calibrated using authentic standard substances for monoaminergic neurotransmitters and their metabolites. The column used was SC-50DS (Eicom), and the mobile phase (pH 3.5) consisted of 83% 0.1 M acetic acid-citric acid buffer, 17% methanol (Nacalai Tesque), 190 mg/L octanesulfonic acid (Nacalai Tesque), and 5 mg/L Na$_2$EDTA (Wako Pure Chemical Ind. Ltd.). The flow rate of the mobile phase was 0.23 ml/min. Monoaminergic neurotransmitters and their metabolites were detected using an electrochemical detector (ECD-300, Eicom) that included a graphite electrode (WE-3G, Eicom). The applied voltage was +700 mV against an Ag/AgCl reference electrode. Data were collected from HPLC via an interface (EPC-300, Eicom) to a personal computer and analyzed using software (PowerChrom, AD instruments Japan, Nagoya).

2.6. Statistical analyses

$P < 0.05$ was considered statistically significant in all statistical analyses.

2.6.1. Ambulatory activity

To eliminate differences in baseline ambulatory activity, the ambulatory activity of each mouse was normalized using the total activity of the mouse during the 30-min adaptation period before administration of drugs. Normalized ambulatory activity was analyzed using the Kruskal-Wallis test followed by the Wilcoxon test, as data were not distributed normally. In the case of the AMPT/RES administration experiment, not only normalized but also actual values of ambulatory activity were analyzed using the Kruskal-Wallis and Wilcoxon tests to examine the effect of AMPT/RES on the baseline ambulatory activity.

2.6.2. c-Fos-IR

Three kinds of statistical analyses were conducted to determine brain region(s) in which c-Fos-IR was involved in the ambulatory response to BUP.

Differences in numbers of c-Fos-IR nuclei in each brain region between saline-administered and BUP-administered mice were
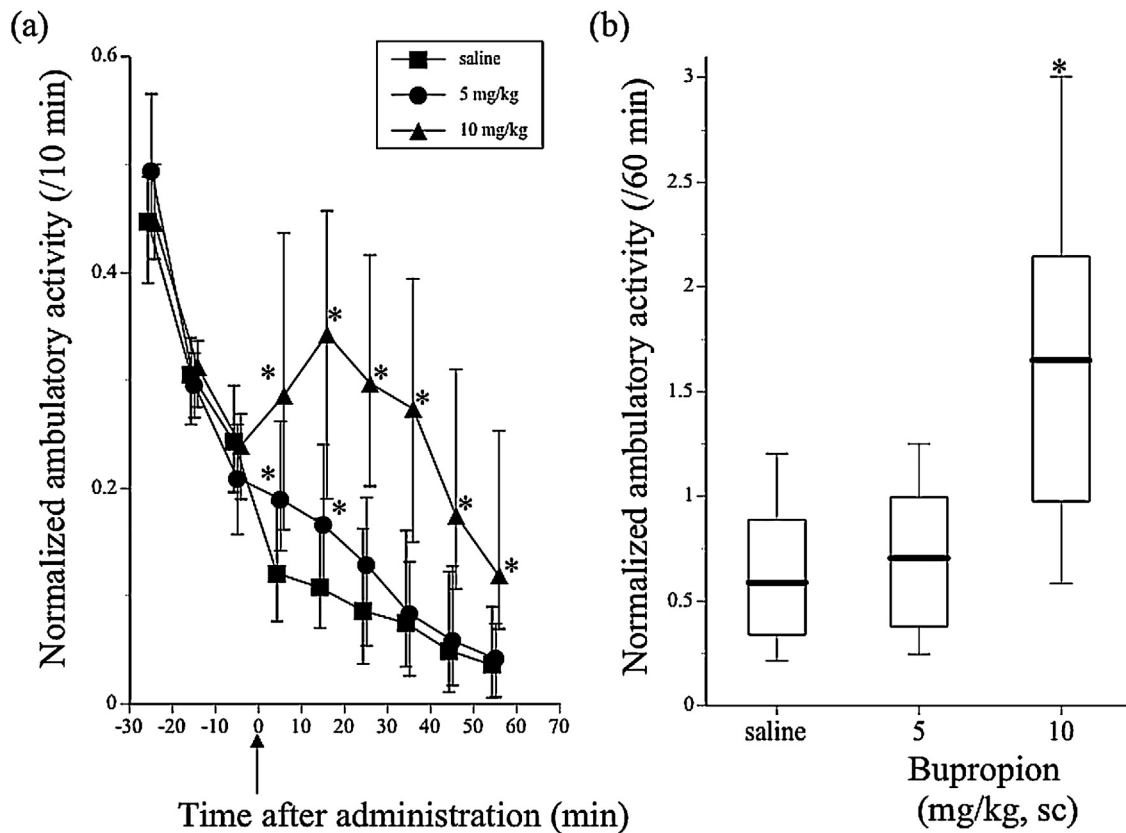
Fig. 1. Effect of bupropion (BUP) on ambulatory activity in ICR mice. The panels show normalized ambulatory activity that was obtained by normalizing actual ambulatory activity to the total ambulatory activity during the 30-min adaptation period before administration in each mouse. (a) Alterations in normalized ambulatory activity before and after subcutaneous administration of saline or 5 or 10 mg/kg BUP. Symbols show median values of normalized ambulatory activity for each 10-min period plotted against the midpoint of the measurement period, and vertical lines denote the first and third quartiles. The arrow indicates the time point for the administration of saline or 5 or 10 mg/kg BUP. (b) Total normalized ambulatory activity for 60 min after administration of saline or 5 or 10 mg/kg BUP. Data are shown using a box plot. *P < 0.05 compared with the saline control. The numbers of animals used were 40–120 (N = 40–120).

compared using the Wilcoxon test, as data were not distributed normally.

Relationships between ambulatory activity and c-Fos-IR in the twenty four brain regions were analyzed using multiple regression analysis [60]. Square root transformations of the c-Fos-IR data and the ambulatory activity were adopted to normalize the data. The step-wise method was first used to select the brain regions in which c-Fos-IR was correlated with ambulatory activity, followed by multiple regression analysis. The best-fit regression equation was obtained by the least-squares method. Significance of the determined multiple regression equation was evaluated using analysis of variance (ANOVA) and R^2 (coefficients of determination), and significance of the partial regression coefficients was tested using the *t*-test.

Furthermore, the relationship between ambulatory activity and c-Fos-IR in each of the seven brain regions identified by the multiple regression analysis was examined using single regression analysis. The best-fit regression equation was obtained by the least-squares method. Significance of the determined single regression equations was evaluated by ANOVA and R^2.

2.6.3. Monoaminergic neurotransmitters and their metabolites

In normal mice, the measured values for monoaminergic neurotransmitters and their metabolites just before administering saline or BUP for each mouse were defined as baseline values, and the measured values before and after administration were expressed as ratios to the baseline values. In the AMPT- and RES-administered mice, actual amounts of monoaminergic

neurotransmitters and their metabolites in dialysates were examined to elucidate the effects of the pretreatment with AMPT and RES on their baseline values and responses of them to BUP.

Measuring 5-HT was difficult because the retention time was close to 25 min in many cases. In addition, as the NE and MHPG measured in the dialysates in the present study were insensitive to pretreatment with AMPT and RES, the measured values for NE and MHPG were believed to be derived from other obstructive sources. Thus, we excluded NE, MHPG, and 5-HT from the examinations. Alterations in DA, DOPAC, HVA, and 5-HIAA in normal mice after administration of saline or BUP were analyzed using two-way repeated ANOVA followed by the Dunnett test that compared BUP data with saline data at each time point. Alterations in DA, DOPAC and HVA after administration of saline or BUP in the mice pretreated with AMPT/RES or saline were analyzed using three-way repeated ANOVA followed by two-way ANOVA, one-way ANOVA, and the Dunnett test.

3. Results

3.1. Effect of BUP on mouse ambulatory activity

When mice were placed into the activity cages, they exhibited high ambulatory activity, followed by a decrease in activity during the 30-min adaptation period. After the adaptation period, saline or 5 or 10 mg/kg BUP was administered to the mice. The ambulatory activity was significantly promoted by administration of 10 mg/kg
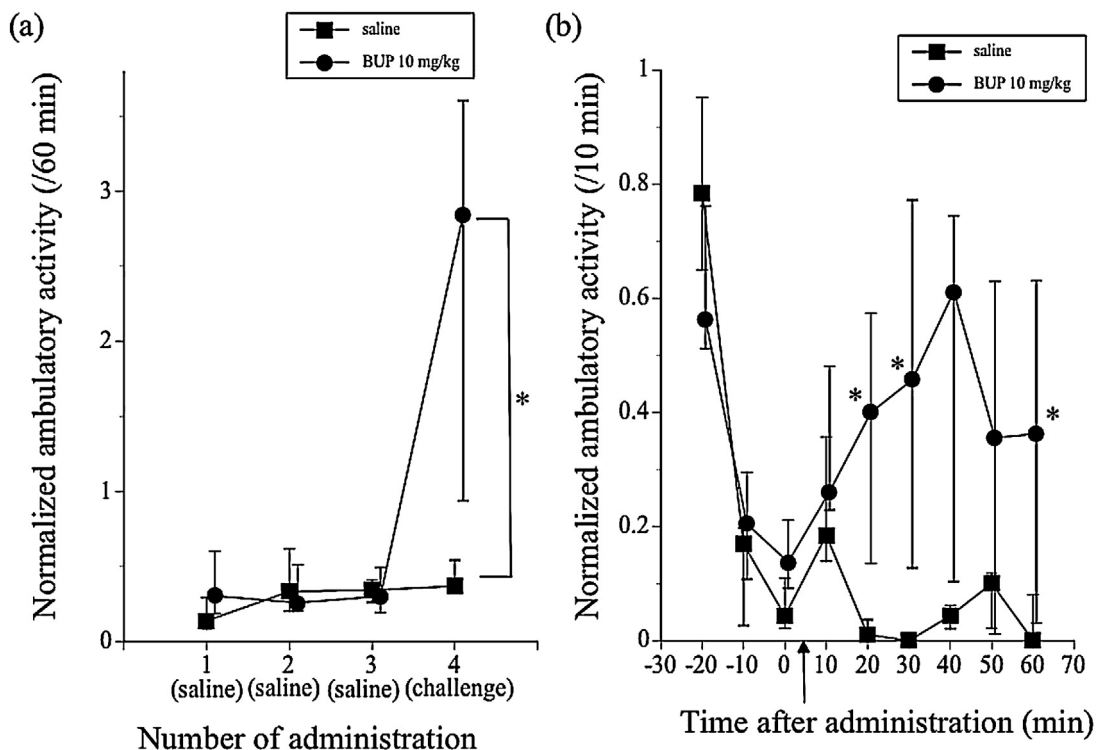
Fig. 2. (a) Daily changes in total ambulatory activity for 60 min after subcutaneous administration of saline or BUP. All mice were given saline on days 1–3 (acclimation session). On day 4 (challenge session), one group was given saline (N = 7), and another group was given 10 mg/kg BUP (N = 10). Symbols show median values of total normalized ambulatory activity for 60 min after administration, and vertical lines denote the first and third quartiles. *P < 0.05 BUP vs. saline on day 4 (challenge session). (b) Time course of ambulatory activity before and after administration of saline or 10 mg/kg BUP on day 4. The arrow indicates time point for the administration. Immediately after the end of the ambulatory measurement, the mice were transcardially perfused with fixative, and their brains were later used for c-Fos immunocytochemistry. Symbols show median values of normalized ambulatory activity for each 10-min period. Symbols were plotted against the midpoint of the measurement period. Vertical lines denote the first and third quartiles. *P < 0.05 BUP vs. saline.
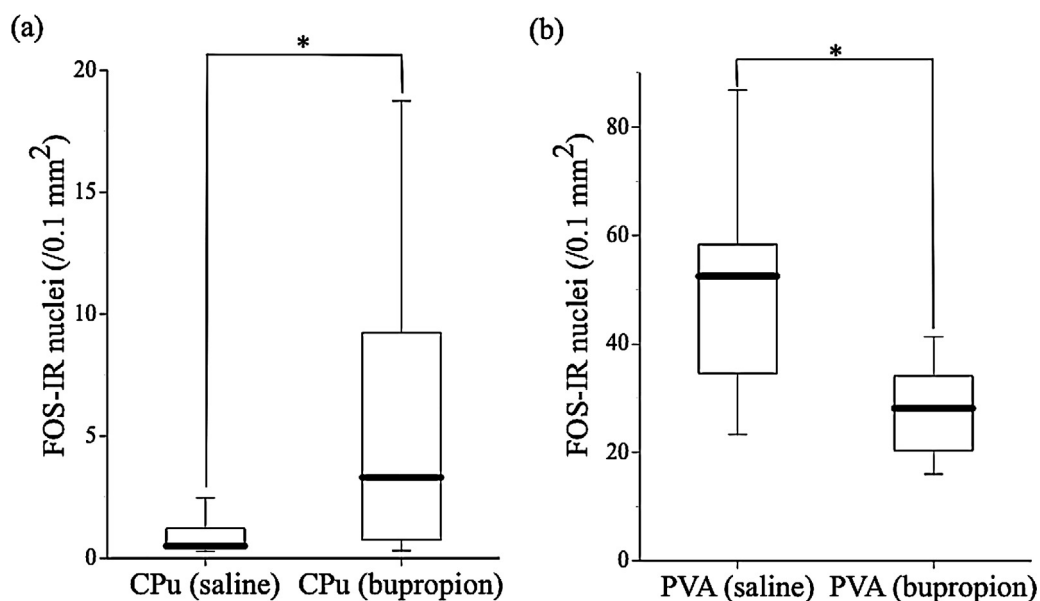

Fig. 3. c-Fos-IR in the CPu (a) and PVA (b) of mice that were given saline or 10 mg/kg BUP. Data are shown using a box plot. *P < 0.05.

BUP (Kruskal-Wallis test; $\chi^2_2 = 64.9426$, P < 0.0001) (Fig. 1(a), (b)). The ambulatory activity reached a maximum 15 min after administration of 10 mg/kg BUP, followed by a decrease in the ambulatory activity to the level of the saline-administered mice within 60 min (Fig. 1(a)).

3.2. Effect of BUP on c-Fos-IR in 24 brain regions and the relationships between ambulatory activity and c-Fos-IR in the brain regions

The ambulatory activity on the 4th day (challenge session) was significantly promoted by 10 mg/kg BUP after 3 consecutive days
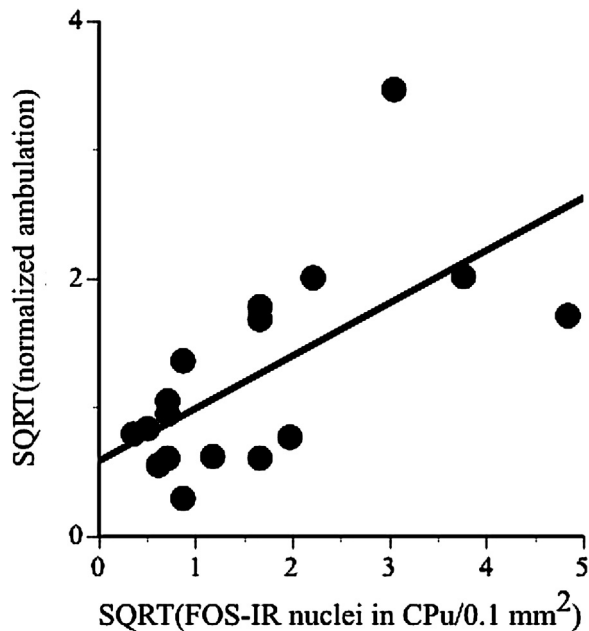
Fig. 4. A result of single regression analysis between ambulatory activity and c-Fos-IR in the CPu. The best-fit regression line was determined using the least-squares method. Statistics for the regression equation are shown in Table 2.

Fig. 5. Effect of pretreatment with saline or α-methyl-*p*-tyrosine (AMPT) + reserpine (RES) on response of ambulatory activity to 10 mg/kg BUP. (a) Changes in actual ambulatory activity. (b) Changes in normalized ambulatory activity. Symbols show the median values of ambulatory activity for each 10-min period that were plotted against the midpoint of the measurement period, and vertical lines denote the first and third quartiles. *P < 0.05 compared with saline control. N = 32–36.

of acclimation ($\chi^2_1 = 8.5714$, P = 0.0034) (Fig. 2(a), (b)). Immediately after the ambulatory measurement, the brains of the mice were fixed by transcardial perfusion of fixative and used for c-Fos immunocytochemistry.

c-Fos-IR nuclei were quantified in 24 brain regions of the mice given saline or BUP. The number of c-Fos-IR nuclei in the CPu of mice that received BUP was significantly larger (Fig. 3(a)), and the number of c-Fos-IR nuclei in the PVA was significantly smaller (Fig. 3(b)) than those of mice that received saline (CPu; $\chi^2_1 = 4.047$, P = 0.0442, PVA; $\chi^2_1 = 5.4857$, P = 0.0192). No significant differences were observed in the numbers of c-Fos-IR nuclei in other brain regions between mice given BUP and saline (data not shown).

The relationships between the ambulatory activity and the numbers of c-Fos-IR nuclei in the 24 brain regions were examined. Seven brain regions were selected by the step-wise method, and multiple regression analysis indicated the following equation:

$$\text{SQRT(normalized ambulation)} = a \times \text{SQRT(c-Fos-IRinCPu)} + b$$
$$\times \text{SQRT(c-Fos-IRinLS)} + c \times \text{SQRT(c-Fos-IRinMnR)} + d$$
$$\times \text{SQRT(c-Fos-IRinLGP)} + e \times \text{SQRT(c-Fos-IRinMGP)} + f$$
$$\times \text{SQRT(c-Fos-IRinLC)} + g \times \text{SQRT(c-Fos-IRinVMH)} + \text{Intercept}$$

where SQRT is square root transformation of the measured value, and a, b, c. . . . . . are partial regression coefficients for the variables. Determined values for the partial regression coefficients and the results of statistical tests for them are shown in Table 1. The multiple regression equation was significant (ANOVA: F(7, 8) = 7.3737, P = 0.0057), and R^2 was 0.865808.

The relationships between the ambulatory activity and the number of c-Fos-IR nuclei in each of the seven brain regions were examined using single regression analysis. The determined regression equations for each of the seven brain regions and their statistics are shown in Table 2. The single regression equation for the CPu was significant (Fig. 4).
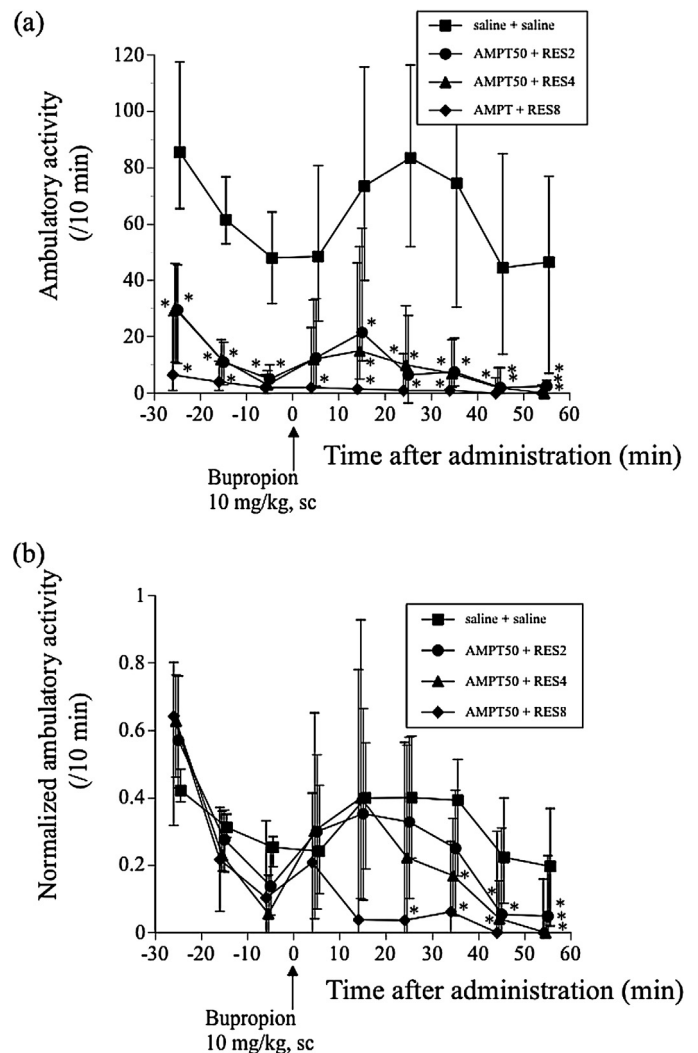
3.3. Effect of pretreatment with AMPT and RES on ambulatory effect of BUP

Pretreatment with 50 mg/kg AMPT and 2–8 mg/kg RES extensively reduced the baseline ambulatory activity (Fig. 5(a)). In addition, the pretreatment also extensively reduced the effect of 10 mg/kg BUP on the actual ambulatory activity (Fig. 5(a)). Response of the normalized ambulatory activity to BUP also significantly decreased depending on time and the dose of RES (at 25 min, $\chi^2_3 = 8.4874$, P = 0.0369: at 35 min, $\chi^2_3 = 15.0212$, P = 0.0018: at 45 min, $\chi^2_3 = 13.3536$, P = 0.0039: at 55 min, $\chi^2_3 = 20.3453$, P = 0.0001) (Fig. 5(b)). Of note, pretreatment with 50 mg/kg AMPT and 8 mg/kg RES abolished the ambulatory response to BUP.

3.4. Effects of drugs that preferentially antagonize DARs on response of ambulatory activity to BUP

Fig. 6 illustrates the dose effects of (a) HAL, (b) CPZ, (c) PIM, (d) FLU, (e) SCH, and (f) SPI on the ambulatory effect of 10 mg/kg BUP. All drugs consistently attenuated the ambulation-promoting effect of BUP. (HAL: $\chi^2_4 = 71.982$, P < 0.0001; CPZ: $\chi^2_4 = 78.4963$,

Table 1
Statistics for each partial regression coefficient obtained by multiple regression analysis for relationships between the ambulatory activity and c-Fos-IR in seven brain regions.

Explanatory variables	Partial regression coefficients	Determined value	t value	Probability
SQRT(c-Fos-IR in the CPu)	a	0.6740728	4.6	0.0018
SQRT(c-Fos-IR in the LS)	b	0.1291112	0.82	0.4363
SQRT(c-Fos-IR in the MnR)	c	0.2988483	2.35	0.0464
SQRT(c-Fos-IR in the LGP)	d	−0.326261	−1.53	0.1637
SQRT(c-Fos-IR in the MGP)	e	0.2327495	2.02	0.0775
SQRT(c-Fos-IR in the LC)	f	−0.371602	−2.43	0.0414
SQRT(c-Fos-IR in the VMH)	g	−0.182014	−2.62	0.0307
Intercept		0.333934	0.73	0.486

Abbreviations: SQRT; square transformation of the measured value, c-Fos-IR; c-Fos-like immunoreactivity, CPu; caudate putamen, LS; lateral septum nucleus, MnR; median raphe nucleus, LGP; lateral globus pallidus, MGP; medial globus pallidus, LC; locus coeruleus, VMH; ventral hypothalamic nucleus.

Table 2
Statistics of single regression equations for relationships between the ambulatory activity and c-Fos-IR in each of seven brain regions.Single regression equation.
SQRT(normalized ambulation) = (Regression coefficient) × (Explanatory variable) + Intercept.

Explanatory variable	Regression coefficient	Intercept	F value	Probability	R^2
SQRT(c-Fos-IR in the CPu)	0.409049	0.5862859	F(1.15) = 10.6043	0.0053	0.414162
SQRT(c-Fos-IR in the LS)	0.0092656	1.2033837	F(1.15) = 0.0037	0.9525	0.000245
SQRT(c-Fos-IR in the MnR)	0.3405928	0.357357	F(1.15) = 4.1809	0.0588	0.217971
SQRT(c-Fos-IR in the LGP)	0.2623237	0.9954338	F(1.15) = 1.5488	0.2324	0.093589
SQRT(c-Fos-IR in the MGP)	0.2928349	0.9146221	F(1.15) = 4.0894	0.0614	0.214223
SQRT(c-Fos-IR in the LC)	0.0956728	1.007142	F(1.14) = 0.2736	0.6091	0.01917
SQRT(c-Fos-IR in the VMH)	−0.036343	1.3765973	F(1.15) = 0.2451	0.6277	0.016079

Abbreviations: SQRT; square transformation of the measured value, c-Fos-IR; c-Fos-like immunoreactivity, CPu; caudate putamen, LS; lateral septum nucleus, MnR; median raphe nucleus, LGP; lateral globus pallidus, MGP; medial globus pallidus, LC; locus coeruleus, VMH; ventral hypothalamic nucleus.
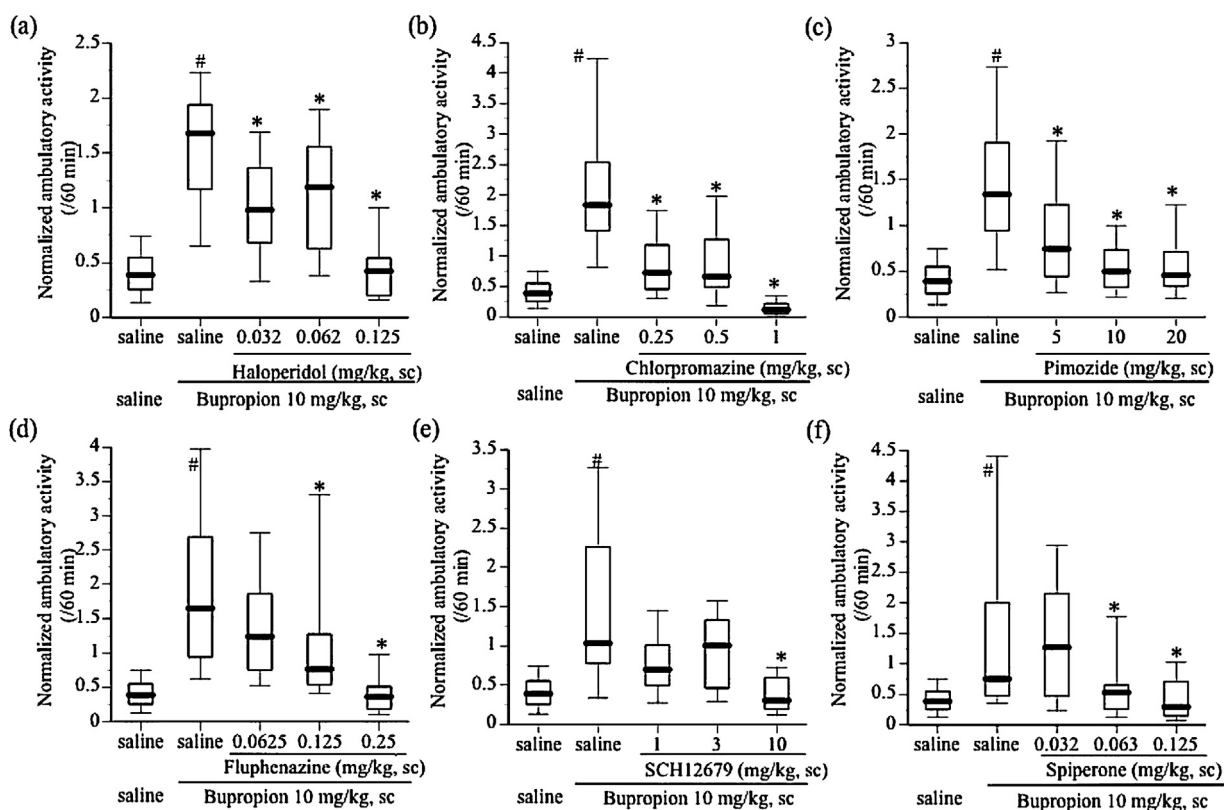

Fig. 6. Effects of 0.032–0.125 mg/kg haloperidol (HAL) (a), 0.25–1 mg/kg chlorpromazine (CPZ) (b), 5–20 mg/kg pimozide (PIM) (c), 0.0625–0.25 mg/kg fluphenazine (FLU) (d), 1–10 mg/kg SCH12679 (SCH) (e), and 0.032–0.125 mg/kg spiperone (SPI) (f) on an effect of 10 mg/kg BUP on ambulatory activity. Data are shown using a box plot on normalized total ambulatory activity for 60 min after administration. (a) N = 20, (b) N = 20, (c) N = 30–40, (d) N = 30–50, (e) N = 10, (f) N = 18. #P < 0.05 compared with saline + saline. *P < 0.05 compared with saline + BUP.

P < 0.0001; PIM: χ^2_4 = 71.7352, P < 0.0001; FLU: χ^2_4 = 118.4002, P < 0.0001; SCH: χ^2_4 = 27.9333, P < 0.0001; SPI: χ^2_4 = 43.8773, P < 0.0001).

3.5. Effects of BUP on extracellular DA, DOPAC, HVA, and 5-HIAA in the CPu

The c-Fos-IR study indicated that the CPu was involved in the ambulatory response to BUP, and the pharmacological studies
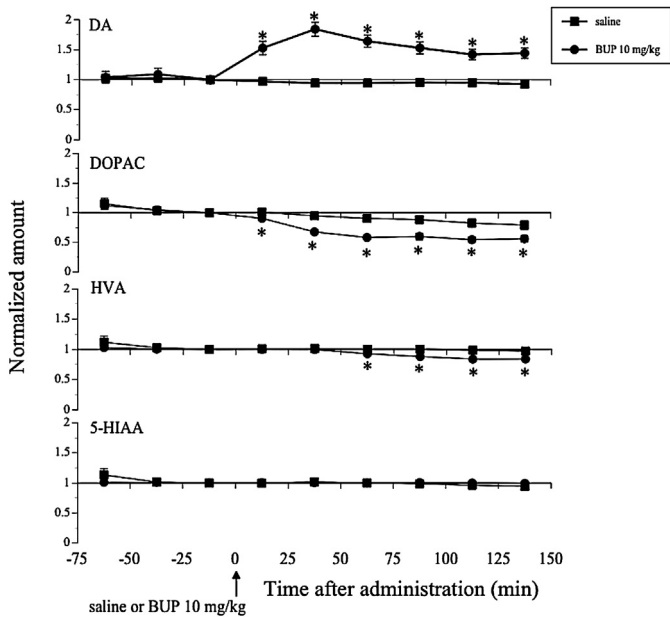
Fig. 7. Alterations in dopamine (DA), its metabolites DOPAC and HVA, and 5-HIAA, a metabolite of serotonin (5-HT), in microdialysis samples obtained from the CPu of mice before and after administration of saline or 10 mg/kg BUP. The actual measured values for each compound just before administering saline or BUP in each mouse were defined as the baseline values, and all measured values were expressed as ratios to the baseline values. Symbols denote mean values that were plotted against the midpoint of the measurement period, and vertical lines indicate standard error of the mean (SEM). *P < 0.05 compared with saline. N = 17–20.

suggested that monoaminergic neurotransmitters, DA in particular, mediated the ambulatory response to BUP. Thus, we examined effects of BUP on extracellular monoaminergic neurotransmitters in the CPu using *in vivo* microdialysis. A dialysis probe was stereotaxically implanted into the CPu (AP: +0.1 mm, ML: +2.0 mm, DV: −2.8 mm) of mice according to the mouse brain atlas [42] (Appendix E).

Fig. 7 illustrates alterations of DA, its metabolites DOPAC and HVA, and 5-HIAA, a metabolite of serotonin (5-HT), in dialysates of the CPu before and after administration of saline or 10 mg/kg BUP. The level of DA in the dialysates significantly increased and reached a maximum at 37.5 min after administration of BUP compared with that after saline administration (repeated-measures ANOVA; dose: $F_{(1, 33)} = 119.1605$, P < 0.0001; time: $F_{(8, 26)} = 18.6742$, P < 0.0001; interaction: $F_{(8, 26)} = 23.1516$, P < 0.0001). Significantly higher levels of DA persisted until 137.5 min after BUP administration. Levels of DOPAC and HVA after BUP administration significantly decreased compared with those after saline administration (DOPAC: dose: $F_{(1, 34)} = 18.726$, P = 0.0001; time: $F_{(8, 27)} = 11.0811$, P < 0.0001; interaction: $F_{(8, 27)} = 9.6829$, P < 0.0001; HVA; dose: $F_{(1, 34)} = 5.5205$, P = 0.0247, time: $F_{(8, 27)} = 5.4972$, P = 0.0004, interaction: $F_{(8, 27)} = 5.4972$, P = 0.0004). On the other hand, the level of 5-HIAA did not alter after BUP administration (dose; $F_{(1,34)} = 0.0222$, P = 0.8825; time; $F_{(8,27)} = 1.7864$, P = 0.124, interaction; $F_{(8, 27)} = 1.2068$, P = 0.3321).

The time course of alteration in ambulatory activity (Fig. 1) was compared with that in the extracellular DA in the CPu (Fig. 7) after administration of BUP or saline (Fig. 8). The ambulatory activity appeared to increase, accompanied by an increase in the extracellular level of DA in the CPu, and the ambulatory activity appeared to decrease to the control level when the extracellular level of DA in the CPu decreased, although the level of DA in BUP-administered mice was significantly higher than that of control mice up to 137.5 min after administration.
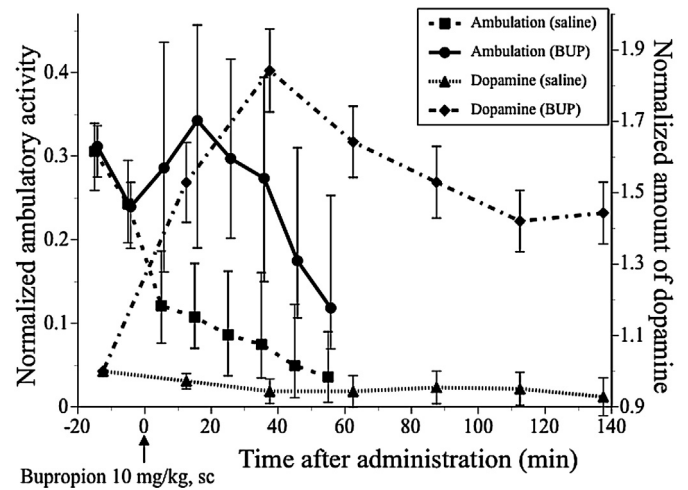

Fig. 8. Comparisons of alterations in ambulatory activity with alterations in DA levels in dialysates of the CPu before and after administration of saline or 10 mg/kg BUP. Data are those shown in Fig. 1 and Fig. 7.
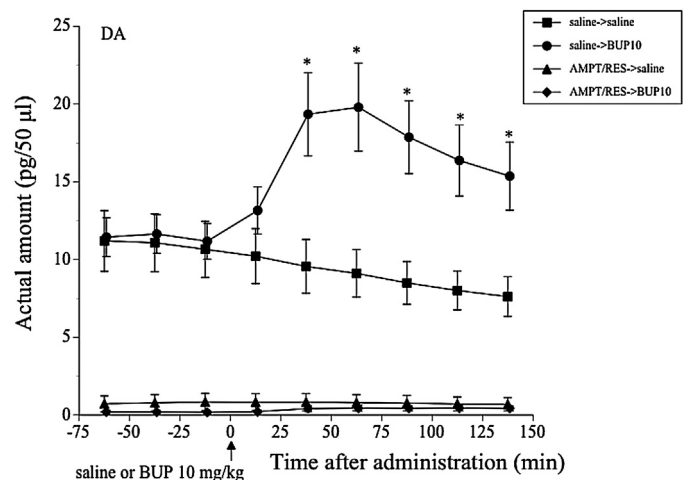

Fig. 9. Alterations in actual measured values of DA in dialysates obtained from the CPu before and after subcutaneous administration of saline or 10 mg/kg BUP. The mice were pretreated with saline or AMPT + RES. Symbols denote the mean values that were plotted against the midpoint of the measurement period, and vertical lines indicate the standard error of the mean (SEM). *P < 0.05 compared with the saline pretreated control. N = 6–8.

3.6. Effect of pretreatment with AMPT and RES on response of extracellular DA in the CPu to BUP

Pretreatment with 50 mg/kg AMPT and 8 mg/kg RES, that remarkably decreased the baseline ambulatory activity (Fig. 5(a)), extensively reduced the actual amounts of DA in the dialysates of the CPu (three-way ANOVA; pretreatment; $F_{(1, 25)} = 79.9599$, P < 0.0001; saline or BUP; $F_{(1, 25)} = 2.727$, P = 0.065; time; $F_{(8, 18)} = 2.9876$, P = 0.0256) (Fig. 9). The actual amount of DA significantly increased after administration of 10 mg/kg BUP in mice pretreated with saline (two-way ANOVA; saline or BUP; $F_{(1, 12)} = 5.3938$, P = 0.0386, time; $F_{(8, 5)} = 1.8636$, P = 0.2554, interaction; $F_{(8, 5)} = 1.99$, P = 0.2326), whereas the actual amounts of DA did not show any significant alterations after BUP administration in mice pretreated with AMPT and RES (two-way ANOVA; saline or BUP; $F_{(1, 12)} = 0.9457$, P = 0.35; time; $F_{(8, 5)} = 1.324$, P = 0.3945, interaction; $F_{(8, 5)} = 0.4796$, P = 0.8302). Thus, pretreatment with 50 mg/kg AMPT and 8 mg/kg RES, that abolished the ambulatory

response to BUP (Fig. 5), abolished the response of extracellular DA in the CPu to BUP.

4. Discussion

This is the first study that examined c-Fos-IR expression in brain of mice to which BUP was administered. In the present study, significant increase in c-Fos-IR relative to saline was observed only in the CPu of mice given 10 mg/kg BUP in the activity cage, though significant increase in c-Fos-IR relative to saline is observed in 42 of 64 brain regions including the CPu and PVA in rats given 20 mg/kg BUP in their home cages [8]. These different results could be due to several factors including differences in experimental conditions, animal species, and dose administered. It is known that AMPH induces c-Fos-IR in brain in different manners depending on the experimental environment [5,39].

Three kinds of analyses for c-Fos-IR (comparison between saline treated group and BUP administered group, multiple regression analysis, single regression analysis) consistently indicated that c-Fos-IR in CPu positively correlated with the ambulatory activity, showing that neuronal activation in the CPu is involved in the ambulatory response to BUP. This result could be due to the characteristic of the tilting cage method that is more sensitive to horizontal movement such as locomotion of mouse in the activity cage. AMPH increases locomotion and c-Fos-IR in the CPu in mice, and the degree of c-Fos-IR in the CPu is positively correlated with the degree of locomotion promoted by AMPH [66]. It is probable that 10 mg/kg BUP principally increases horizontal movement of mouse in the activity cage through activating the CPu.

Co-administration of AMPT and RES abolished the ambulatory response to BUP. Furthermore, HAL, CPZ, PIM, FLU, SCH, and SPI reduced the ambulatory response to BUP. These pharmacological studies indicate that monoaminergic neurotransmitters, DA in particular, mediate the ambulatory response to BUP. Then, the present study examined effects of BUP on monoaminergic neurotransmitters in the CPu, as the c-Fos-IR study showed the CPu is involved in the ambulatory response to BUP. BUP significantly increased extracellular DA level in the CPu at the dose that promoted the ambulatory activity. The ambulatory activity appeared to increase, accompanied by an increase in the extracellular level of DA in the CPu, and the ambulatory activity appeared to decrease to the control level when the extracellular level of DA in the CPu decreased. The pretreatment with AMPT and RES, that abolished the ambulatory response to BUP, abolished the response of DA in the CPu to BUP. These findings show that DA in the CPu is involved in the ambulation-promoting effect of BUP. DAT is expressed in the mouse CPu [49], suggesting that BUP increases extracellular level of DA primarily through inhibiting DAT in the mouse CPu as in the case of the rat CPu [33,37,38]. Increase of extracellular level of DA must increase c-Fos-IR in the CPu in mice given BUP as DA induces c-Fos-IR through DARs [64,36,15].

The number of c-Fos-IR was significantly smaller in the PVA, however, c-Fos-IR in the PVA was not correlated with the ambulatory response to BUP. The results suggest that BUP affects the PVA functions [4,40] but the PVA is not related to the ambulatory response to BUP. Multiple regression analysis indicated that the ambulatory activity was a function of c-Fos-IR not only in the CPu but also in the LS, MnR, LGP, MGP, LC, and VMH. These brain regions interact one another. For example, the LGP and MGP receive neuronal input from the CPu that is known as an indirect pathway from the CPu to the SNR. The indirect pathway mediates the locomotor effect of AMPH in mice [24]. The CPu receives neuronal input from the LC and MnR in mice [19,35]. Interactions among these brain regions may also be involved in the ambulatory response to BUP.

Multiple regression analysis suggests that neuronal activity in the LC, the origin nucleus of NE neurons, is involved in the ambulatory response to BUP. The mouse CPu receives NE input from the LC and expresses NET [19,45]. BUP inhibits NET [16]. NE induces c-Fos through NE receptors [9] that are expressed in the CPu of mice [20]. NE may also play some roles for the effects of BUP on the neuronal activity in the CPu and the ambulatory activity, however, the current microdialysis study unfortunately failed to elucidate reliable responses to BUP of NE and its metabolite MHPG in the CPu. The results are probably due to low levels of NE and MHPG in the mouse CPu and the low IC_{50} of BUP for inhibiting NET [16]. BUP did not affect extracellular level of 5-HIAA, a metabolite of serotonin (5-HT), in the CPu in mice similar to in rats [11,33,37]. As increase of extracellular DA was accompanied by decrease of extracellular HVA and DOPAC in mice given BUP, BUP may not affect serotonergic state in the CPu in mice.

Striatal DA system is involved in the locomotion-promoting effects of AMPH and MetAMPH in mice [7,24,36,51,64], and sensitization to the locomotor effects of AMPH and MetAMPH has been well studied as animal models of AMP and/or MetAMPH psychosis [28,44]. On the other hand, the "endogenous sensitization" hypothesis of schizophrenia has been proposed. The hypothesis postulates that a sensitized DA system is intrinsic to the disease and is responsible for the genesis of psychotic symptoms [1,12,32,34]. The hypothesis is supported by the reports that demonstrate enhanced striatal DA release induced by an acute AMPH challenge in first-episode schizophrenia patients relative to healthy controls [1,12,32] and overexpression of mesolimbic D2 receptors in the patients [2,31,63]. As BUP produces sensitization to the ambulation-promoting effect in mice [58] and the present study revealed that the DA in the CPu is involved in the ambulation-promoting effect of BUP, the concern about the potential of BUP to increase a risk of psychosis [29] seems reasonable.

In conclusion, the present study elucidated that DA in the CPu is involved in the ambulation-promoting effect of BUP in mice. The results of the present study suggest that other brain regions such as the LS, MnR, LGP, MGP, LC and VMH and another neurotransmitter NE also play some roles for the ambulatory response to BUP, however, elucidating the roles must await further research.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxrep.2016.06.005.

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