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Enhancement of the rewarding effects of 3,4-methylenedioxymethamphetamine in orexin knockout mice

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Running title: Orexin depletion and psychostimulants

ABSTRACT

Orexinergic neurons, which are closely associated with narcolepsy, regulate arousal and reward circuits through the activation of monoaminergic neurons. Psychostimulants as well as 5-HT-related compounds have potential in the treatment of human narcolepsy. Previous studies have demonstrated that orexin receptor antagonists as well as orexin deficiencies affect the pharmacological effects of psychostimulants. However, little information is available on the consequences of psychostimulant use under orexin deficiency. Therefore, the present study was designed to investigate the abuse liability of psychostimulants in orexin knockout (KO) mice. In the present study, conditioned place preferences induced by methamphetamine and methylphenidate were not altered in orexin

KO mice. Interestingly, we found that MDMA induced a conditioned place preference in orexin KO mice, but not in wild type (WT) mice. In addition, MDMA produced methylphenidate/methamphetamine-like discriminative stimulus effects in orexin KO mice, but not WT mice. Increases in 5-HT and dopamine release in the nucleus accumbens induced by MDMA were not altered by knockout of orexin; the steady-state level of G protein activation was higher in the limbic forebrain of orexin KO mice. In substitution tests using a drug discrimination procedure, substitution of 5-HT_{1A} receptor agonist for the discriminative stimulus effects of methylphenidate was enhanced in orexin KO mice. These findings indicate that the orexinergic system is involved the rewarding effects of psychostimulants. However, there is a risk of establishing rewarding effects of psychostimulants even under orexin deficiency. On the other hand, deficiencies in orexin may enhance the abuse liability of MDMA by changing a postsynaptic signal transduction accompanied by changes in discriminative stimulus effects themselves.

Abbreviations: MDMA, 3,4-methylenedioxy-methamphetamine; KO, knockout; WT, wild type; 5-HT, serotonin; DEPC, diethylpyrocarbonate; ERK, extracellular-regulated kinase

Keywords: MDMA, orexin, 5-HT, rewarding effects, discriminative stimulus effects,

1. Introduction

Excessive daytime sleepiness including intrinsic sleep disorders like narcolepsy can have detrimental consequences [1]. Activation of monoaminergic neurons produces robust changes across sleep-wake states, whereas orexin, which is expressed exclusively in the perifornical area, dorsomedial as well as lateral hypothalamus, plays an important role in sleep-wake states [2-5]. It has been recognized that dysfunction of the orexin system is critically linked to human narcolepsy [6-9]. Orexin-containing neurons project to practically all brain regions, including monoaminergic nuclei such as those in the locus coeruleus (noradrenaline), raphe nuclei (serotonin: 5-HT) and ventral tegmental area (dopamine), and positively regulate these neurons [10,11]. Thus, dysfunction of monoaminergic systems regulated by orexinergic neurons might be involved in narcolepsy.

Psychostimulants (e.g., methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA) and methylphenidate) that increase the level of monoamines within the synaptic cleft produce behavioral activation such as arousal, alertness [1], reinforcing effects and subjective effects in humans [12]. We previously demonstrated that orexinergic systems regulate the activation of dopaminergic systems. Further, morphine-induced rewarding effects were significantly suppressed in orexin knockout (KO) mice [13]. More recently, we have shown that hyperlocomotion induced by methylphenidate (dopamine and noradrenaline uptake inhibitor) or methamphetamine (monoamine releaser) was reduced in orexin KO and orexin/ataxin-3 mice, whereas hyperlocomotion induced by MDMA (DA and 5-HT releaser) was increased in both types of mice to a greater degree than in wild type (WT) mice [14]. 5-HT_{1A} receptor agonist as well as 5-HT_2 receptor agonist inhibited a decrease in locomotor activity of orexin KO mice [15]. These findings suggest that depletion of orexin differentially regulates dopaminergic and serononergic neurons, and that 5-HT receptor agonists may have therapeutic potential for narcolepsy.

Even though psychostimulants have strong abuse potential and have been used for the treatment of narcolepsy, there is little information available regarding the abuse liability of psychostimulants in orexin-deficient conditions. Importantly, previous reports have indicated that the orexinergic system participates in the addictive response by regulating the mesolimbic dopaminergic system [16-18]. Furthermore, an orexin receptor antagonist would have significant potential for treatment of psychostimulant abuse [19]. However, the potential risk for the development of psychostimulant abuse in narcolepsy has not been clarified. Therefore, the major aim of the present study was to investigate the effects of depletion of orexin on the acquisition of the rewarding effects of psychostimulants using the conditioned place preference paradigm in mice. This information may give us a better understanding of the abuse liability of psychostimulants during treatment of narcolepsy.

2 Material and methods

2.1. Animals

Male orexin KO mice with a mixed C57BL/6 J-129/SvEv background, which completely lack the prepro-orexin gene, were generated using a previously described method [20],

and maintained at 22-25 g (80 % free-feeding weight) for the drug discrimination study. Heterozygous male and female mice were mated to obtain null (−/−) mutants and WT (+/+) littermates. The genotype of orexin KO mice was identified by conventional PCR with the following primers; neo primer, 5'- CCGCTATCAGGACATAGCGTTGGC-3', genomic primer, 5'-GACGACGGCCTCAGACTTCTTGGG-3', and genomic primer, 5'-TCACCCCCTTGGGATAGCCCTTCC-3'. Animals were housed in a room maintained at 23 ± 1°C with a 12 h light/dark cycle (lights on 8:00 A.M. to 8:00 P.M.). The mice used in this study were males over 15 weeks old. Food and water were available ad libitum, except in the drug discrimination study. All experiments were conducted during the light periods.

2.2. Drugs

The drugs used in the present study were racemic MDMA hydrochloride, methylphenidate hydrochloride (Sigma-Aldrich Co., St Louis, MO), (±)-8-hydroxy-2-(di-n-propylamino)tetralin hydrobromide (8-OH-DPAT; Sigma-Aldrich Co.), DOI (Sigma-Aldrich Co.), and methamphetamine hydrochloride (Dainippon-Sumitomo Pharmaceutical Co. Ltd, Osaka, Japan). MDMA was synthesized by the Bureau of Social Welfare and Public Health, Tokyo Metropolitan Government. All of the drugs were dissolved in saline and administered s.c. in a volume of 0.1 ml/kg. All doses refer to the salt forms of the drugs.

2.3 Place conditioning

Place-conditioning studies were conducted based on previous report [21] using a shuttle box (15 × 30 × 15 cm: w × l × h) composed of an acrylic resin board divided into two equal-sized compartments. One compartment was white with a textured floor and the other was black with a smooth floor. The place-conditioning schedule consisted of three phases (pre-conditioning test, conditioning, and post-conditioning test). The pre-conditioning test was performed as follows: the partition separating the two compartments was raised to 7 cm above the floor, a neutral platform was inserted along

the seam separating the compartments, and mice that had not been treated with either drugs or saline were then placed on the platform. The time spent in each compartment during a 900-s session was recorded automatically using an infrared beam sensor (KN-80, Natsume Seisakusyo Co. Ltd, Tokyo, Japan). Conditioning sessions (3 for drug: 3 for saline) were conducted once daily after the pre-conditioning test. Immediately after the injection of drugs, the animals were placed in the compartment opposite that in which they had spent the most time in the pre-conditioning test for 1 h. On alternate days, these animals were treated with saline and placed in the other compartment for 1 h. On the day after the final conditioning session, a post-conditioning test that was identical to the pre-conditioning test was performed.

Our previous study showed that hyperlocomotion-induced by MDMA was enhanced, whereas that induced by methamphetamine and methylphenidate was suppressed in orexin-deficient mice [14]. Therefore, we expected that methamphetamine and methylphenidate-induced place preferences would be suppressed in orexin KO mice. Previous reports have demonstrated that methamphetamine and methylphenidate produce robust behavioral effects including conditioned place preference in rodents [14,22-24]. Therefore, the dose of methamphetamine (2 mg/kg) and methylphenidate (10 mg/kg) to produce place preference in mice was selected based on these reports.

2.4. Mouse in vivo microdialysis study and quantification of dopamine and 5-HT

Microdyalysis procedure was conducted based on previous research [25]. Stereotaxic surgery was performed under sodium pentobarbital (50 mg/kg) anesthesia. Mice were placed in a stereotaxic apparatus and the skull was exposed. A small hole was then made using a dental drill. A microdialysis probe (D-I-6-01; 1 mm membrane length; Eicom) was inserted in the nucleus accumbens (from bregma: anterior, +1.5 mm; lateral, -0.9 mm; ventral, -4.9 mm) according to the atlas of [26]. The probe was secured with cranioplastic cement. At 24 h after surgery, mice were placed in the experimental cages (30 cm wide × 30 cm long × 30 cm high). The microdialysis probe was perfused with an artificial CSF (aCSF) (0.9 mM $MgCl_2$, 147.0 mM NaCl, 4.0 mM KCl, and 1.2 mM CaCl2) continuously at a flow rate of 2 μl/min. Outflow fractions were taken every 20 min. After 15 baseline fractions were collected, mice were given MDMA (5 mg/kg) or saline (1 ml/kg). For this experiment, dialysis samples were collected for 180 min after MDMA or

saline treatment. Dialysis fractions were then analyzed using HPLC with ECD (HTEC-500; Eicom). Dopamine and 5-HT were separated by a column with a mobile phase containing 0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4, 2.0 mM sodium 1-decane sulfonate, 0.1 mM EDTA (2Na), and 1% methanol. The mobile phase was delivered at a flow rate of 550 μl/min. Dopamine and 5-HT were identified according to the retention times of dopamine and 5-HT standards, and the amounts of dopamine and 5-HT were quantified by calculations using peak areas. The baseline microdialysis data were calculated as concentrations in the dialysates. Other microdialysis data are expressed as percentages of the corresponding baseline level.

Determination of the location of the infusion cannula placement and drug diffusion was performed at the completion of the experiments. Mice were deeply anesthetized with sodium pentobarbital (50 mg/kg) at the end of the experiment and given microinjections of ink for anatomical localization of cannula sites (0.3 μl). The brain was then removed by decapitation and cut into coronal sections. Cannula placement was mapped onto a stereotaxic atlas [26] and confirmed to be in the nucleus accumbens. Mice with cannulas in the wrong location were excluded from the study.

2.5. RNA Isolation and Reverse Transcription-polymerase Chain Reaction (RT-PCR)

Brains were quickly retrieved and dissected to give 2 mm-thick slices of the limbic forebrain, including the nucleus accumbens and lower midbrain, containing the ventral tegmental area with brain blocker. The samples were stored at -80 ℃ until use. RT-PCR including the design of primers was conducted based on previous report [15]. Briefly, first-strand cDNA was synthesized from 1 μg of total RNA. Total RNA was incubated at 70 °C for 10 min with oligo $(dt)_{12–18}$ 1 μl and water that had been treated with diethylpyrocarbonate (DEPC) 67 μl, and then quickly cooled on ice. The RNA sample was then treated with 10 × RT buffer 10 μL, 0.1 M dithiothreitol 10 μl, 25 mM $MgCl_2$ 10 μl, and 10 mM dNTP Mix 1 μl (Invitrogen Life Technologies, Co.), incubated at 42 °C for 10 min, and then reverse-transcribed with 50U reverse transcriptase II (RT-II; Invitrogen Co., Tokyo, Japan) at 42 °C for 45 min. The enzyme was heat-inactivated at 70 °C for 10 min. In RT-PCR, Go Taq® Green Master Mix (Promega, Co., WI) assays

were run on a thermocycler of D_1-receptor, D_2-receptor, 5-HT_{1A}-receptor, 5-HT_{2A}-receptor, 5-HT_{2C}-receptor, and β-actin mRNA. The PCR reaction mixtures contained 2× Go Taq® Green Master mix 15 µl, primers at the adopted concentrations 2 µl, cDNA 30 ng, and DEPC-treated water up to 30 µl. RT-PCR was conducted at 95 °C for 2 min, followed by 30 cycles of 94 °C for 30 s and 55 °C for 1 min, and 72 °C for 1 min. The PCR products were resolved by electrophoresis in 2 % agarose gel with a DNA ladder marker. The intensity of PCR fragments visualized by ethidium bromide staining was quantified with a FluorChem3 system (Laboratory & Medical Supplies, Tokyo, Japan). The data were normalized with respect to the β-actin level.

Primers:

D_1-receptor GenBank™ accession No. NM010076, (sense 1489-1508): 5'-CCT CCC TGA ACC CCA TTA TT-3' and (antisense 1817-1836): 5'-GGG TAA CGG GTT GGA TCT TT-3'

D_2-receptor GenBank™ accession No. NM010077, (sense 278-297): 5'-GAG AAG GCT TTG CAG ACC AC-3' and (antisense 565-584): 5'-AGG ACA GGA CCC AGA CAA TG-3'

5-HT_{1A}-receptor GenBank™ accession No. NM008308, (sense 1306-1325): 5'-CCC CCC AAG AGC CTG AA-3' and (antisense 1621-1640: 5'-GGC AGC CAG AGG ATG AA-3'

5-HT_{2A}-receptor GenBank™ accession No. NM172812, (sense 1848-1867): 5'-TCA CCT ACT TCC TGA CTA TC-3' and (antisense 2452-2471): 5'-TGT CTG TAC ACA TCT CTT CC-3'

5-HT_{2C}-receptor GenBank™ accession No. NM008312, (sense 715-732): 5'-CTC ACT CCT TGT GCA CCT-3' and (antisense 979-999): 5'-CCC ACC AGC ATA TCA GCA ATG-3'

β-actin GenBank™ accession No. NM007393, (sense 282-301): 5'-CCC AGA GCA AGA GAG GTA TC-3' and (antisense 602-621): 5'-AGA GCA TAG CCC TCG TAG AT-3'

2.6. Guanosine-5′-o-(3-thio) triphosphate ([^{35}S]GTPγS) binding assay

GTPγS binding assay was conducted based on previous study [25]. For membrane preparation, sections of the mouse limbic forebrain and frontal cortex were quickly removed after decapitation and rapidly transferred to a tube filled with ice-cold buffer. The membrane homogenate (30 μg protein/assay) was prepared as described previously [22] and incubated at 37°C for 30 min in 1 ml of assay buffer with various concentrations of each agonist, 300 μM guanosine-5′-diphosphate (GDP) and 100 pM [^{35}S]GTPγS (specific activity, 1.250 Ci/mmol; Perkin Elmer, Waltham, MA). The reaction was terminated by filtration using Whatman GF/B glass filters (Brandel, Gaithersburg, MD) presoaked in 50 μM Tris–HCl, pH 7.4, and 5 μM $MgCl_2$ at 4 °C for 2 h. The filters were washed three times with 5 ml of an ice-cold Tris–HCl buffer, pH 7.4, and then transferred to scintillation-counting vials. Next, 3 ml of clear-sol 2 (Nacalai Tesque Inc., Kyoto, Japan) was added to the vials and equilibrated for 12 h, and the radioactivity in the samples was measured with a liquid scintillation analyzer. Specific binding values were estimated by subtracting the value of [^{35}S]GTPγS in the presence of 10 μM unlabeled GTPγS.

2.7. Drug discrimination studies

Experiments were conducted in operant-conditioning chambers (model ENV-307; Med Associates, St. Albans, VT) equipped with two nose-poke holes and a food cup mounted midway between the holes. Yellow lamps were installed above each of the holes. Chambers were enclosed within sound- and light-attenuating boxes and supplied with white noise (80db) to mask extraneous sound inside the box. Reinforcement consisted of 20-mg food pellet (Bio-Serv, Frenchtown, NJ). Following steps were conducted based on our previous study using rats [24]. Before mice were trained to discriminate between methylphenidate and saline, all mice were trained to nose-poke a hole. Mice were trained to nose-poke either the right or left hole in the daily sequence LRRLLRRLLR (R = right, L = left). Training began under a reinforcement schedule of fixed ratio 1 (FR 1), in which the mouse was presented with a food pellet each time it nose-poked a hole.

When reinforcement was provided, the light in the hole was illuminated. The FR requirement for food reinforcement was gradually increased to a value of 10. After the response rates had stabilized under FR 10, mice were trained to discriminate between 5.0 mg/kg of methylphenidate and saline. In the discrimination training, methylphenidate

(M) and saline (S) were administered in a session-to-session sequence of MMSS (double alternation schedule), and the assignment of left and right holes to drug and saline states was counterbalanced. Mice were required to respond on the stimulus-appropriate hole to obtain reinforcement; there were no programmed consequences for responding on the incorrect hole. Substitution tests were only performed after the discrimination criterion described below had been satisfied for at least five consecutive daily discrimination-training sessions (accuracy of at least 83% and fewer than 12 responses to obtain the first reinforcement). These criteria are based on our previous paper [24].

After the animals' performance reached the criterion, a dose-response or substitution test was initiated; test sessions were performed only when first reinforcement was obtained with ≤ 12 responses for at least three consecutive discrimination training sessions. In the dose-response test, mice were placed in the operant box until they had made 10 responses on either hole or 5 min had elapsed. In the substitution test, the treatment time and doses of drugs used were as follows: 15 min for 0.625 – 5.0 mg/kg of methylphenidate, 0.25-1.0 mg/kg of methamphetamine, 1.25–5.0 mg/kg of MDMA, 0.3-3.0 mg/kg of 8-OH-DPAT and 0.5-2.0 mg/kg of DOI. If the mice did not make 10 responses during each component, the response was judged to have been disrupted.

During training sessions, accuracy was defined in terms of the number of correct responses as a percentage of the total responses before the first food pellet. During the test sessions, performance was expressed in terms of the number of drug-appropriate responses as a percentage of the total responses upon completion of FR 10. Drugs were considered to have substituted for the discriminative stimulus effects of the training drugs if more than 80% of the responses were on the drug-appropriate nose-poke, while a lack of substitution was defined as less than 50% of responses on the drug-appropriate nose-poke. The response rate was calculated as the total number of responses before the completion of 10 responses on either lever divided by the time (minutes) taken to complete the first ratio.

2.8. Statistical analysis

Data are expressed as the mean with S.E.M. The statistical significance of differences between groups was assessed by the Mann-Whitney test or one-way and two-way

ANOVA followed by the Bonferroni multiple comparisons test. All statistical analyses were performed using Prism software (version 5.0a, GraphPad Software). A P value of <0.05 was considered to reflect significance.

2.9. Ethical considerations

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals (Hoshi University), as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Every effort was made to minimize the number of animals used in the following experiments (e.g., n = 8 and optimized doses were used).

3 Results

3.1. Evaluation of the acquisition of conditioned place preferences with drugs of abuse in orexin knockout mice

The deletion of orexin gene was confirmed by genotype PCR (Fig. 1A), and there was no difference in preconditioning scores among the different genotypes. Our unpublished study demonstrated that 1 – 10 mg/kg of MDMA induced neither place preference nor place aversion in ddY mice under the exact same conditions as in our previous research [25]. In this study, MDMA (1 - 10 mg/kg) also did not produce either place preference or place aversion in WT mice ($F(3,31)=1.359$, $P=0.2756$). In contrast, a significant place preference was observed in orexin KO mice under the administration of MDMA (1 - 10 mg/kg) ($F(3,31)=6.294$, $P<0.0021$) (WT mice) (Fig. 1D). In the present study, methamphetamine (1 mg/kg) ($F(3,31)=12.1$, $P<0.0001$) and methylphenidate (10 mg/kg) ($F(3,31)=20.66$, $P<0.0001$) induced place preferences in both WT and orexin KO mice (Fig. 1B and C).

3.2. Neurochemical changes in the limbic forebrain in orexin KO mice.

To elucidate the effects of KO of orexin on the MDMA-induced release of 5-HT and dopamine from nerve terminals, we also measured the release of 5-HT and dopamine in the nucleus accumbens with an in vivo microdialysis procedure using WT and orexin KO mice. Five mg/kg of MDMA was selected based on previous studies that monitored the

enhancement of monoamine release in orexin KO mice [29]. Basal extracellular levels of 5-HT and dopamine in the nucleus accumbens were 3.16 ± 0.01 nM and 4.50 ± 0.04 nM/20 min in WT, and 3.15 ± 0.01 nM and 4.49 ± 0.01 nM/20 min in orexin KO mice (mean with S.E.M. of five samples). MDMA significantly increased the release of 5-HT ($F(1,143)=13.04$, $P<0.0001$ (WT mice); $F(1,104)=21.95$, $P<0.0001$ (orexin KO mice)) and dopamine ($F(1,143)=28.49$, $P<0.0001$ (WT mice); ($F(1,104)=30.739$, $P<0.0001$ (orexin KO mice)) into the nucleus accumbens, whereas MDMA had no effects on 5-HT and dopamine metabolites (Fig. 2) in orexin KO or WT mice. Therefore, we hypothesized that KO of orexin may affect 5-HTnergic or dopaminergic functions at the postsynaptic level. There were no differences between WT and orexin knockout mice regarding 5-HT_{1A}-, 5-HT_{2A}-, 5-HT_{2c}-, D_1- and D_2-receptor mRNA levels in the limbic forebrain including the nucleus accumbens (Fig. 3A-E) and midbrain (data not shown) as evaluated using RT-PCR. On the other hand, steady-state levels of G-protein activation in orexin KO mice in the limbic forebrain were significantly higher than those in WT mice ($F(1,43)=5.06$, $P<0.01$)(Fig. 3D), whereas no difference was observed in the G-protein levels in the prefrontal cortex between WT and orexin KO mice (data not shown), indicating that depletion of orexin may alter postsynaptic function in the nucleus accumbens.

3.3. MDMA substitutes for the discriminative stimulus effects of methylphenidate in orexin KO mice

One of the most important determinants of the abuse potential of drugs is the nature of their subjective effects in humans. The drug discrimination procedure has considerable potential for the evaluation of subjective effects in animals. To further understand the behavioral changes produced by MDMA in orexin KO mice, the drug discrimination procedure was used in mice trained to discriminate between methylphenidate and saline. Approximately 36 sessions were required for methylphenidate (5 mg/kg)-saline discrimination in WT mice, whereas 32 sessions were required in orexin KO mice. Once mice attained the criterion, drug-saline discrimination was stabilized and maintained with a high degree of accuracy. During the dose-response tests, methylphenidate (1.25-5.0 mg/kg) produced a dose-related increase in methylphenidate-appropriate responses in all of the mice (Fig. 4A). In substitution tests, methamphetamine (0.25-1.0 mg/kg)

substituted for the discriminative stimulus effects of methylphenidate in both WT and orexin KO mice (Fig. 4B). Consistent with previous results in rats [23], MDMA (1.25-5.0 mg/kg) did not produce methylphenidate-appropriate responding in WT mice (less than 50 %) (Fig. 4C). The dose-response curve of MDMA for the discriminative stimulus effects of methylphenidate differed between WT and orexin knockout mice ($F(2,41)=5.176$, $P<0.0282$), and methylphenidate-appropriate responding with 5 mg/kg of MDMA was significantly higher in orexin KO mice (more than 80%) than in WT mice (Fig. 4C). While the response rates were decreased at 5 mg/kg of MDMA, neither behavioral changes nor behavioral disruption were observed during the substitution test.

The 5-HT_2-receptor agonist DOI did not engender methylphenidate-appropriate responding in either strain of mice (Fig. 4D). Similarly, the 5-HT_{1A}-receptor agonist 8-OH-DPAT did not produce methylphenidate-associated responses in WT mice. In contrast, orexin KO mice showed a significant upward shift of the dose-response curve of 8-OH-DPAT for the discriminative stimulus effects of methylphenidate in compared with WT mice ($F(2,40)=8.273$, $P<0.0064$), and this difference was significant at 3 mg/kg (Fig. 4E).

4. Discussion

Orexin neurons project to the ventral tegmental area, which is a region closely related to rewarding processes, and administration of orexin A into the ventral tegmental area activates the dopaminergic system [16]. Orexin receptor antagonists suppress the development [16] and expression [30,31] of behavioral sensitization to locomotor activity induced by psychostimulant drugs, expression of a cocaine-induced conditioned place preference [32] and the self-administration of cocaine [33]. Furthermore, systemic administration as well as microinjection of orexin receptor antagonist into the ventral tegmental area reduces the increase in dopamine release from the nucleus accumbens induced by cocaine [33]. On the contrary, methamphetamine and methylphenidate could induce the place preferences in orexin KO mice. With regard to these discrepant results, we found that dopamine turnover was decreased whereas 5-HT turnover was increased in the limbic forebrain of orexin deficient mice [14], indicating that hypofunction of the dopaminergic system in orexin-deficient mice could theoretically induce a compensatory increase in serotonergic systems. It is well known that 5-HT as well as noradrenergic

system also contributes to the behavioral effects of psychostimulants. Thus, orexinergic system has a role in the rewarding effects of psychostimulants, whereas compensatory effects may explain why the rewarding effects of psychostimulants were established in orexin deficient conditions.

In a self-administration study [34], MDMA was self-administered, but was not a potent reinforcer; MDMA itself induces a taste aversion [35]. Whereas, conditioned place preference induced by MDMA could been established in mice [36, 37]. In the present study, MDMA did not induce a place preference in WT mice, indicating that MDMA has lower rewarding efficacy than other psychostimulants in our present conditions. In the present study, we also found that the rewarding effects induced by MDMA could be enhanced in orexin KO mice, and MDMA substituted for the discriminative stimulus effects of methylphenidate in orexin KO mice, but not WT mice. Thus, the discriminative stimulus effects of MDMA in orexin KO mice could not be differentiated from other psychostimulant-like effects, and such a change may affect the establishment of the rewarding effects of MDMA in orexin KO mice.

Harper et al. [38] showed that a high dose of MDMA itself could shift its discriminative stimulus effects from MDMA-like effects to amphetamine-like effects in rats that were trained to discriminate between MDMA, amphetamine and saline using a 3-lever discrimination procedure. In addition, a relatively high dose of MDMA induces a conditioned place preference [39]. Thus, monoaminergic tone through the release of neurotransmitters or the activation of monoamine receptors induced by MDMA may explain why the behavioral effects induced by MDMA were enhanced in orexin KO mice. However, the increase in 5-HT as well as dopamine release induced by MDMA was not different in orexin KO mice. Our previous study demonstrated that hyperlocomotion induced by MDMA, but not those induced by methamphetamine and methylphenidate, was enhanced in orexin KO mice [14]. In contrast, enhanced locomotor activity produced by MDMA in orexin KO mice was completely suppressed by either 5-HT_{1A} receptor or 5-HT_2 receptor antagonist [14]. These findings suggest that sensitization through postsynaptic 5-HT receptor related signaling is involved in the enhanced behavioral responses caused by MDMA in orexin KO mice. Therefore, we focused on postsynaptic function in orexin-deficient conditions.

We have demonstrated that 5-HT_{1A} or 5-HT_2 receptor agonists, but not methamphetamine and methylphenidate, substituted for the discriminative stimulus effects of MDMA in rats [15], indicating that activation of the serotonergic system 5-HT_{1A} and 5-HT_2 receptors is crucial for the discriminative stimulus effects of MDMA. In substitution tests, there was no difference in the effects of the 5-HT_2 receptor agonist between orexin KO and WT mice trained to discriminate between methylphenidate and saline. Furthermore, the 5-HT_{1A} receptor agonist 8-OH-DPAT did not produce methylphenidate-like responding in WT mice, however, methylphenidate-like responding induced by 8-OH-DPAT was significantly enhanced in orexin KO mice. These findings suggest that functional changes through 5-HT_{1A} receptor-mediated signaling in orexin-deficient conditions may affect the pharmacological effects induced by 5-HT-related compounds. Therefore, we finally focused on the postsynaptic functions in the limbic forebrain under orexin KO conditions based on the fact that 5-HT_{1A} and 5-HT_2 receptors are G-protein-coupled receptors [40.41]. Although no changes were observed in the mRNA levels of 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2c} in the limbic forebrain of orexin KO mice as compared with WT mice, the steady-state level of G-protein activation in the limbic forebrain in orexin KO mice was higher than that in WT mice. Our findings may imply that responsiveness to MDMA could be sensitized at the serotonergic postsynaptic levels by the depletion of orexin as a compensatory effect [14,15].

In summary, orexin-deficient conditions could not abolish the development of rewarding effects induced by psychostimulants. On the other hand, deficiencies in orexinergic systems positively regulate the abuse liability of MDMA by conversion into methylphenidate-like effects. The orexinergic system is involved the rewarding effects of psychostimulants, however our findings suggest that there is a risk of establishing rewarding effects of psychostimulants even under orexin deficiency.

Authorship contribution statement

Mori T: Study design, data analysis, interpretation of data and drafting the manuscript.

Uzawa N, Hirayama S, Iwase Y, Hokazono M, Udagawa Y, Masukawa D: Data acquisition and analysis, interpretation of findings.

Suzuki T: Supervised the study.

Conflict of interest and disclosure

The authors have nothing to disclose for this study.

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Fig. 1 Genotype PCR of WT and orexin KO mice gives 400 bp and 600 bp bands, respectively (a). Methamphetamine (METH; 2 mg/kg; b)-, methylphenidate (MPH; 10 mg/kg; b)- and MDMA (1.0 - 10 mg/kg; c)-induced place preference in wild-type (WT) and prepro-orexin knockout (KO) mice using the conditioned place preference paradigm. Ordinate, Preference for the drug-paired place, as defined by the post-conditioning test score minus the pre-conditioning test score on the drug-treatment side. Each point represents the mean conditioning score with S.E.M. of 8 mice. *P < 0.05, **P < 0.01, versus saline-treated mice.

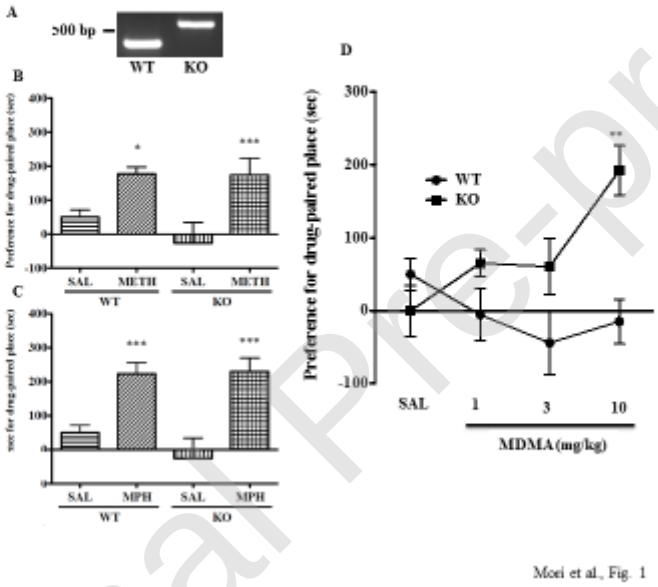


Fig. 2. Effects of treatment with MDMA on the dialysate levels of dopamine (a) or 5-HT (d) and its metabolites (b, c, e) in the nucleus accumbens in wild-type (WT) and prepro-orexin knockout (KO) mice. MDMA (5mg/kg) or saline (Sal) was injected at time 0. The data are expressed as percentages of the corresponding baseline levels with S.E.M. of 5 mice. *P < 0.05, **P < 0.01 and ***P < 0.001 versus saline-treated mice. Histological representations of probe placement in the nucleus accumbens and striatum (f). Coronal sections are reproduced from Paxinos and Franklin (2004). Vertical bars represent the 2-mm exposed membrane of each microdialysis probe.

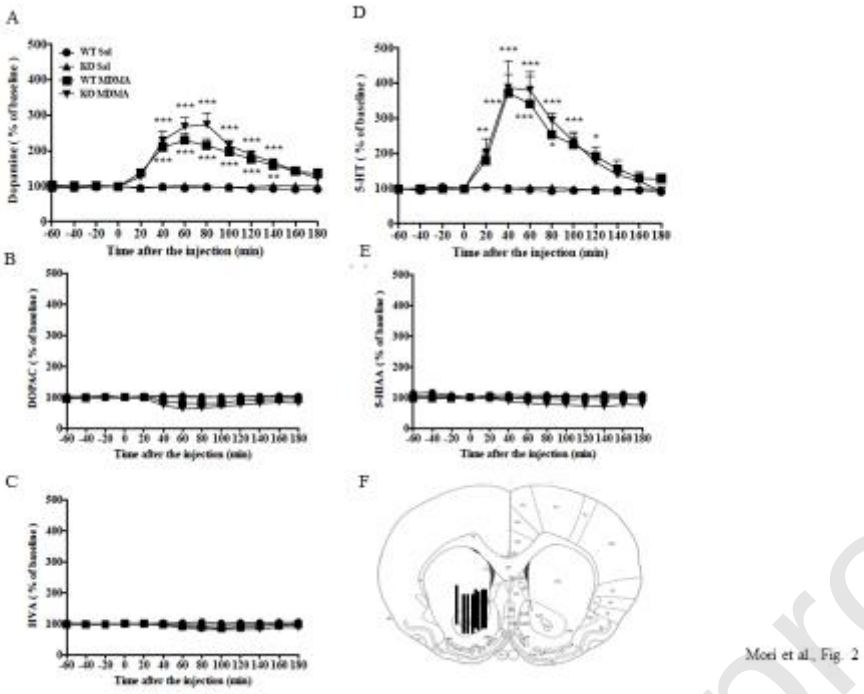


Fig. 3. Changes in 5-HT_{1A} receptor (a), 5-HT_{2A} receptor (b), 5-HT_{2C} receptor (c), D_1 receptor (d) and D_2 receptor (e) mRNA expression in the limbic forebrain of wild-type (WT) and prepro-orexin KO mice. Upper panels: Representative RT-PCR for 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2C}, D_1, and D_2 receptor mRNA in the limbic forebrain of WT and orexin KO mice. Bottom panels: The values for each mRNA were normalized by the value for β-actin mRNA as an internal standard. Each column represents the mean ± S.E.M. of 5 samples. Changes in postsynaptic events in orexin knockout mice. Specific binding of [^{35}S]γGTP to each brain region membrane of wild-type (WT) and prepro-orexin knockout (KO) mice (f). Each column represents the mean ± S.E.M. of 5-6 samples. **P < 0.01, versus WT mice.

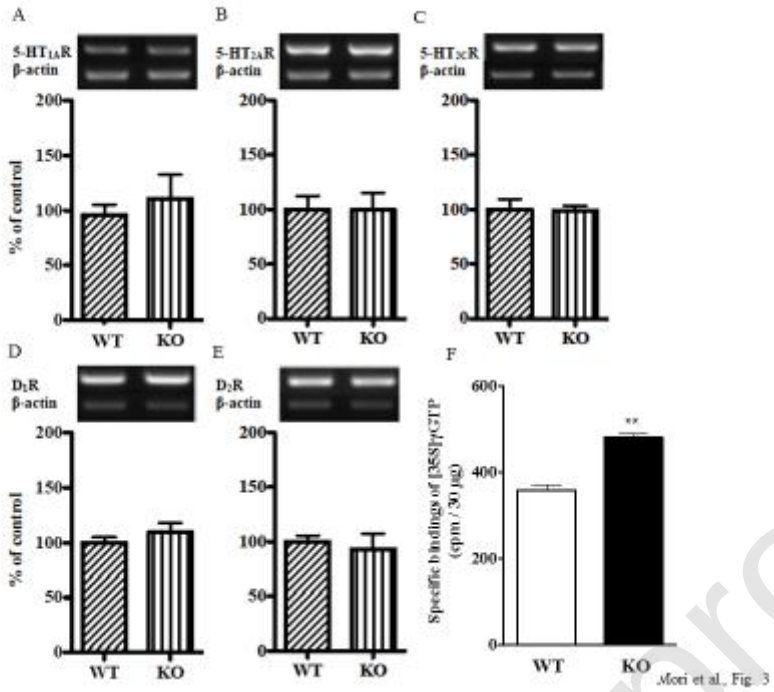
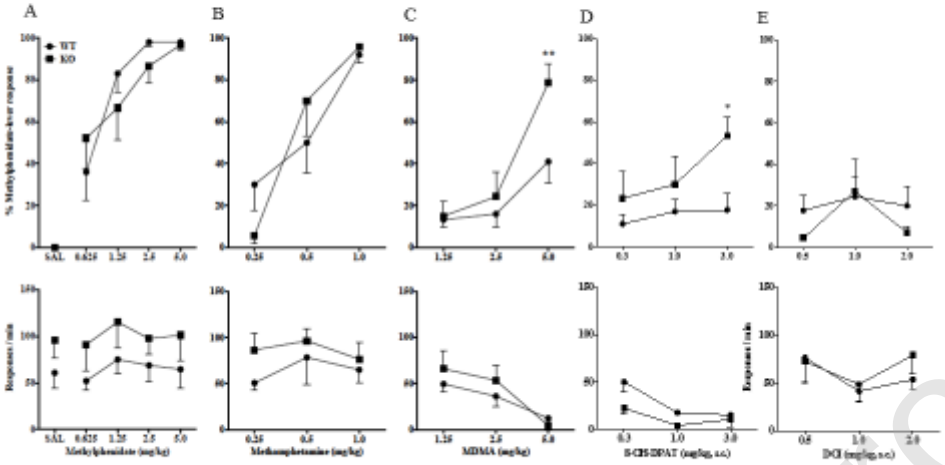


Fig. 4. Dose-response (a) and substitution tests of methamphetamine (b), MDMA (c), 8-OH-DPAT (d) and DOI (e) for the discriminative stimulus effects of methylphenidate in wild-type (WT) and prepro-orexin knock-out (KO) mice that had been trained to discriminate between 5 mg/kg methylphenidate and saline. Each point represents the mean percentage of methylphenidate-appropriate responding (top panel) and the mean response rates (bottom panel) with S.E.M. of 8 animals. *P < 0.05, **P < 0.01 versus WT mice.



Moei et al., Fig. 4