An Antihyperkinetic Action by the Serotonin 1A–Receptor Agonist Osemozotan Co-administered With Psychostimulants or the Non-stimulant Atomoxetine in Mice

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Abstract. It has been demonstrated that treatment of hyperactive mice with psychostimulants produced a calming effect depending on serotonergic neurotransmission. Our previous study also showed that hyperactivity in mice lacking pituitary adenylate cyclase–activating polypeptide (PACAP) was ameliorated by amphetamine in a serotonin (5-HT)1A-dependent manner and that amphetamine calmed wild-type mice given the 5-HT1A agonist 8-OH-DPAT. Here, we examined if 5-HT1A–mediated pathways can be a determinant of the action of other psychostimulants as well as the non-stimulant atomoxetine by examining locomotor activity in mice co-administered with the 5-HT1A agonist osemozotan. Co-administration of osemozotan with either methamphetamine or amphetamine was not only antihyperkinetic, but also decreased locomotion to below basal levels. In contrast, osemozotan just nullified methylphenidate-induced hyperactivity. The non-stimulant atomoxetine did not induce hyperactivity, but co-administration of atomoxetine with osemozotan produced a calming effect. The adjunctive effect of osemozotan added to the psychostimulants was blocked by the 5-HT1A antagonist WAY-100635 at a low dose (0.1 mg/kg), suggesting the involvement of a presynaptic 5-HT1A–mediated mechanism. However, WAY-100635 (up to 1 mg/kg) did not block the effect of atomoxetine plus osemozotan. The present results may provide insights into the therapeutic mechanisms of the psychostimulants and atomoxetine for hyperkinetic disorders.

Keywords: atomoxetine, attention-deficit/hyperactivity disorder (ADHD) medication, locomotor activity, psychostimulant, serotonin 5-HT1A receptor

Introduction

Although psychostimulants such as amphetamine (AMP) and methamphetamine (MAMP) induce hyperactivity in both animals and humans, psychostimulant treatment has long been recognized to attenuate hyperactivity, paradoxically, in certain hyperactive conditions in humans such as attention-deficit/hyperactivity disorder (ADHD) (1–3). It has been recently demonstrated that treatment of hyperactive mice with psychostimulants produced a calming effect depending on serotonergic neurotransmission (4). However, the underlying mechanisms remain to be elucidated.

We recently demonstrated that mice lacking pituitary adenylate cyclase–activating polypeptide (PACAP, PACAP−/− mice), a neuropeptide with pleiotropic activities (5), exhibit marked phenotypes including a high

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early mortality rate before weaning, hypophagia, and leanness, as well as behavioral abnormalities such as hyperlocomotion and jumping behavior in an open field and increased novelty-seeking behavior (6–8). Interestingly, hyperactive behavior in these mice was ameliorated by AMP, and this paradoxical antihyperkinetic effect was completely blocked by the serotonin (5-HT)1A (5-HT1A)-receptor antagonist WAY-100635. In these mutant mice, the hypothermic response to 5-HT1A agonists such as 8-OH-DPAT and buspirone was significantly impaired, and it is suggested that auto-inhibitory 5-HT1A–receptor function is reduced, leading to consequent changes in 5-HT transmission. Therefore, we further examined if AMP could produce an antihyperkinetic effect in wild-type normal mice receiving 8-OH-DPAT and observed a calming effect of this drug combination (7).

Because it has been hypothesized that psychostimulants exert rate-dependent effects that show an inverse correlation with the baseline rate of initial locomotor activity (1), our results implied that 5-HT1A-mediated pathways can be important determinants of the psychostimulant-elicited, rate-dependent effects on locomotor activity. In our aforementioned study, we only examined the effect of AMP, but there are other psychostimulants that have to be examined, including MAMP, methylphenidate (MPH), and the first non-stimulant medication atomoxetine (ATX) (9), which was recently approved by the FDA (U.S. Food and Drug Administration) to treat ADHD in both children and adults. Therefore, in the present study, we examined the effects of these drugs co-administered with the selective 5-HT1A agonist osemozotan (OSE) (10,11) on open field locomotor activity in normal ICR mice.

**Materials and Methods**

**Animals**

All animal experiments were carried out in accordance with protocols approved by the Animal Research Committee of Osaka University. Male Slc:ICR (Institute of Cancer Research) mice were obtained from Japan SLC (Shizuoka) at 6 weeks of age, and were allowed to acclimate in our animal facility for at least 1 week before initiation of experiments. The number of mice used for each treatment group was indicated in the figure legends, and each animal was used for only one treatment. All mice were housed in a temperature (23 ± 1°C)- and light-controlled room with a 12-h light / 12-h dark cycle (lights on from 8:00 AM to 8:00 PM) and allowed free access to water and a standard diet, except during behavioral testing.

**Drugs**

OSE (previously called MKC-242 and also known as MN-305; (S)-5-[3-[(1,4-benzodioxan-2-ylmethyl)amino]propoxy]-1,3-benzodioxole HCl; Mitsubishi Tanabe Pharma Corp., Yokohama) was suspended at a concentration of 0.03 and 0.1 mg/ml in 0.5% carboxymethylcellulose (Nacalai Tesque, Kyoto) and injected intraperitoneally in a volume of 10 ml/kg of body weight 10 min before the test began. WAY100635 {N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-[2-(pyridinyl)cyclohexanecarboxamide; Sigma-Aldrich Inc., Tokyo} was dissolved at concentrations of 0.02 and 0.2 mg/ml in saline (0.9% NaCl solution) and injected subcutaneously in a volume of 5 ml/kg of body weight 30 min before the test. MAMP (Dainippon Pharma, Osaka), AMP (Takeda Chemical Industries, Ltd., Osaka), MPH (Novartis Pharma, Tokyo), and ATX (Mitsubishi Tanabe Pharma Corp.) were dissolved at concentrations of 0.01–1 mg/ml in saline and injected intraperitoneally in a volume of 10 ml/kg of body weight just before the test. GBR12909 (Sigma-Aldrich) and fluvoxamine (Solvay Seiyaku KK, Tokyo) were dissolved at concentrations of 0.03–0.3 and 1–3 mg/ml, respectively, in saline and injected intraperitoneally in a volume of 10 ml/kg of body weight just before the test began.

**Open field locomotor activity**

Locomotor activity was quantified using an infrared photocell beam detection system Acti-Track (Panlab, Barcelona, Spain). Following injection of drug or an equal amount of corresponding vehicle solution, the mice were placed in plastic activity monitoring boxes (45 cm-wide × 45 cm-deep × 30 cm-high) and tracked for 60 min, with data being stored permanently; parameters indicative of locomotor activity, such as distance traveled, were assessed. Each mouse was tested individually and had no contact with the other mice. The box was cleaned between tests.

**Statistical analyses**

The statistical significance of differences was assessed by one-way analysis of variance (ANOVA), followed by post hoc Fisher’s least significance difference tests. Statistical significance was defined as $P<0.05$. All values are expressed as means ± S.E.M.

**Results**

**Antihyperkinetic or calming effects of MAMP co-administered with OSE in mice**

To confirm the involvement of 5-HT1A signaling in the antihyperkinetic effect of ADHD medications, we have examined the effects of psychostimulants co-
administered intraperitoneally with the selective 5-HT\textsubscript{1A} agonist OSE on open field locomotor activity in normal mice. OSE at 0.3 and 1 mg/kg body weight did not affect locomotor activity (Fig. 1). The effects of MAMP co-administered with OSE are shown in Fig. 2. One-way ANOVA revealed a significant effect of treatment \[ F(7,101) = 24.51, P<0.0001 \]. Post-hoc analysis indicated that MAMP at doses of more than 0.3 mg/kg, but not 0.1 mg/kg, induced hyperactivity. When 1 mg/kg OSE was co-administered, the hyperactivity induced by MAMP at 0.3 and 1 mg/kg was significantly reduced. Interestingly, co-administration of 1 mg/kg OSE and 1 mg/kg MAMP markedly reduced locomotor activity to below vehicle-treated baseline levels; hereafter, we use the term ‘calming effect’ to mean the effect lowering locomotor activity to below vehicle-treated baseline levels and use the term ‘antihyperkinetic effect’ to indicate the effect lowering locomotor activity down to the vehicle-treated baseline level but not below it. Lower doses of OSE (0.3 mg/kg) did not affect MAMP-induced hyperactivity.

**Antihyperkinetic effects by AMP, MPH, and ATX co-administered with OSE**

The paradoxical calming effect seen with MAMP plus OSE was also observed following administration of AMP plus OSE (Fig. 3a). One-way ANOVA revealed a significant effect of treatment \[ F(2,18) = 23.68, P<0.0001 \]. Post-hoc analysis indicated that AMP (2 mg/kg) caused significant hyperactivity, but when co-administered with OSE (1 mg/kg), it induced a calming effect. AMP at 1 mg/kg, but not 0.3 mg/kg, either alone or in combination with 1 mg/kg OSE, similarly induced hyperactivity or a calming effect, respectively (data not shown). The effects of MPH co-administered with OSE are shown in Fig. 3b. One-way ANOVA revealed a significant effect of treatment \[ F(2,15) = 14.11, P<0.001 \]. Post-hoc analysis indicated that MPH (10 mg/kg) caused significant hyperactivity as well. Although OSE significantly blocked the MPH-induced hyperactivity, the combination, OSE plus MPH, did not induce a calming effect. Figure 3c shows the effect of ATX co-administered with OSE. One-way ANOVA revealed a significant effect of treatment \[ F(2,15) = 6.41, P<0.01 \]. Post-hoc analysis indicated that the non-stimulant ADHD medicament ATX (1 mg/kg) did not change locomotor activity, whereas co-administration of ATX and OSE significantly reduced locomotor activity.

The 5-HT\textsubscript{1A} antagonist WAY-100635 blocks the antihyperkinetic/calming effect of OSE co-administered with MAMP or MPH, but not ATX

WAY-100635 was tested at a low dose (0.1 mg/kg), which antagonizes somatodendritic 5-HT\textsubscript{1A} autoreceptors in the raphe nucleus, and at a higher dose (1 mg/kg), which completely blocks postsynaptic 5-HT\textsubscript{1A} receptors.
The effects of WAY-100635 either alone or in combination with MAMP are shown in Fig. 4, a and b. One-way ANOVA revealed a significant effect of treatment \( F(5,26) = 18.12, P < 0.0001 \). Post-hoc analysis indicated that neither dose of WAY-100635 affected locomotor activity or MAMP (1 mg/kg)-induced hyperactivity. Figure 4c shows the effect of WAY-100635 on the calming effect of OSE co-administered with MAMP. One-way ANOVA revealed a significant effect of treatment \( F(4,51) = 81.38, P < 0.0001 \). Post-hoc analysis indicated that WAY-100635, either at 0.1 mg/kg or 1 mg/kg, significantly inhibited the calming effect.
induced by MAMP plus OSE. Figure 5a shows the effect of WAY-100635 on the antihyperkinetic effect of OSE co-administered with MPH. One-way ANOVA revealed a significant effect of treatment \[F(4,23) = 10.13, P < 0.0001\]. Post-hoc analysis indicated that either dose of WAY-100635 effectively inhibited the antihyperkinetic effect induced by MPH plus OSE. The effects of WAY-100635 on the calming effect of OSE co-administered with ATX are shown in Fig. 5b. One-way ANOVA revealed a significant effect of treatment \[F(4,21) = 5.46, P < 0.01\]. Post-hoc analysis indicated that WAY-100635 (at either 0.1 or 1 mg/kg) caused no significant inhibition of the calming effect induced by ATX plus OSE.

The adjunctive effects of OSE plus the selective dopamine transporter inhibitor GBR12909 or the selective serotonin reuptake inhibitor fluvoxamine

As the transporters for the monoamines dopamine, norepinephrine, and 5-HT are targets for psychostimulants (15, 16), we examined the adjunctive effect of OSE when co-administered with the selective dopamine transporter inhibitor GBR12909 or with the selective serotonin-reuptake inhibitor fluvoxamine. Figure 6a shows the effect of OSE plus GBR12909. One-way ANOVA revealed no significant effect of treatment \[F(6,72) = 1.89, P = 0.09\]. The effects of OSE plus fluvoxamine are shown in Fig. 6b. One-way ANOVA revealed a significant effect of treatment \[F(4,44) = 3.71, P < 0.05\]. Post-hoc analysis indicated that fluvoxamine at 10 and 30 mg/kg per se slightly reduced initial locomotor activity; however, when co-administered with OSE, this reduction was slightly more prominent, but it induced a flat body posture, a 5-HT syndrome.

**Discussion**

The present study demonstrated that the 5-HT\(_{1A}\) agonist OSE co-administered with either the psychostimulants MAMP, AMP, and MPH or the non-stimulant ATX produce antihyperkinetic and/or calming effects in mice. Although psychostimulants are widely prescribed for the treatment of ADHD, their antihyperkinetic or calming effects are not easily understood, and the underlying physiological mechanisms remain to be elucidated (1, 2). The mechanism by which ATX, a selective norepinephrine-reuptake inhibitor, exerts its effects in ADHD is also largely unknown (9). Moreover, the pathomechanisms involved in ADHD remain unknown. It has been hypothesized that psychostimulants increase or decrease activity or attention depending on baseline activity levels; thereby stimulants produce an inverted U-shaped dose–response influence on behavior (rate-dependent effects of stimulants) (1, 3). Previous reports show that a 5-HT\(_{1A}\) agonist such as 8-OH-DPAT...
attenuates psychostimulant-induced behavioral sensitization and increases in locomotor activity over control levels (17–20).

We previously showed that PACAP−/− mice show hyperactivity that is decreased by AMP and that this paradoxical antihyperkinetic effect depends on 5-HT₁A− receptor signaling, as it was completely blocked by WAY-100635 (7). The present results, taken together with these previous findings, suggest that 5-HT₁A− receptor–mediated pathways are involved in the rate-dependent effects elicited by psychostimulants, in that the activation of 5-HT₁A receptors may result in a leftward parallel shift of the inverted U-shaped dose–response relationship of psychostimulants.

The calming effect of OSE co-administered with MAMP, AMP, or ATX was remarkable, lowering the locomotor activity to below vehicle-treated baseline levels. OSE has been developed as a selective 5-HT₁A− receptor ligand and a candidate anxiolytic and antidepressant by us (10, 11) and is currently under clinical trial for generalized anxiety disorder and insomnia. OSE has a pharmacological feature of full and partial agonism at presynaptic and postsynaptic 5-HT₁A receptors, respectively (10). OSE decreases cortical 5-HT release in a presynaptic 5-HT₁A receptor–mediated manner, while it increases dopamine release in the prefrontal cortex and hippocampus, but not in the nucleus accumbens or striatum. The latter action of OSE is supposed to be a postsynaptic 5-HT₁A receptor–mediated response (21). The regionally distinct dopamine release induced by OSE may explain its antihyperkinetic effects when co-administered with psychostimulants.

The antihyperkinetic effect of OSE co-administered with MPH (10 mg/kg) is contrary to the calming effect of OSE co-administered with the other drugs. Therefore, we examined the effects of a wide range of doses of MPH and found that OSE plus MPH at 1 and 30 mg/kg could similarly induce the antihyperkinetic effect (unpublished data). MAMP, AMP, and MPH potently inhibit the dopamine transporter and the norepinephrine transporter with similar Ki values (0.26–0.56 and 0.12–0.19 µM, respectively). In contrast, although MAMP and AMP moderately inhibit the 5-HT transporter (Ki value: 9.3 and 24 µM, respectively), MPH inhibits the most weakly among the three drugs (114 µM) (15, 16). Such a difference in this pharmacological property of MPH, that is, relative inhibition of the three transporters, may explain the different effects of MPH.

The present observation that ATX per se did not significantly change locomotor activity is in line with an earlier study that demonstrates that ATX did not alter extracellular levels of dopamine in striatum or nucleus accumbens (22).

Relatively low doses of WAY-100635 (for example, 0.1 mg/kg) are known to block presynaptic somatodendritic 5-HT₁A autoreceptors in the raphe nuclei (12), while high doses of WAY-100635 (e.g., 1 mg/kg) completely block postsynaptic 5-HT₁A receptors (13, 14). The present observation that the calming effect elicited by OSE co-administered with MAMP or MPH was completely blocked by WAY-100635 at both 0.1 and 1 mg/kg suggests that this behavioral action may be a consequence of the activation of presynaptic 5-HT₁A autoreceptors or the activation of both presynaptic and postsynaptic 5-HT₁A receptors. In addition, the observation that WAY-100635 at doses up to 1 mg/kg did not block the effect of atomoxetine plus osemozotan suggests different mechanisms of action between the psychostimulants and ATX, although the underlying mechanisms remain unclear at present.

In order to dissect the mechanisms underlying the antihyperkinetic effect of OSE co-administered with psychostimulants, we examined the adjunctive effect of OSE with the selective dopamine-transporter inhibitor GBR12909 or with the selective serotonin-reuptake inhibitor fluvoxamine. However, neither GBR12909 nor fluvoxamine co-administered with OSE produced a calming effect, as seen with MAMP and AMP, although OSE plus fluvoxamine reduced initial locomotor activity and were associated with flat body posture immediately after drug administration. These results are in good contrast with the observation that the selective norepinephrine transporter inhibitor ATX clearly produced a calming effect when co-administered with OSE. These data suggest the mechanistic importance of norepinephrine-transporter inhibition for the observed calming effect.

The present results support the possibility that hyperkinetic behavior might be controlled through precise targeting of 5-HT receptors (4), suggesting that 5-HT₁A− receptor agonists adjunctive to psychostimulants, as well as the non-stimulant ATX, may be promising agents for pharmaceutical intervention in hyperkinetic disorders. Although we should carefully consider species differences in brain function before drawing conclusions about the situation for humans, the present results may provide insight into the treating mechanism of ADHD medications that may involve 5-HT₁A receptor–mediated signaling.

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