

*Short Communication***Sustained Exposure to 3,4-Methylenedioxymethamphetamine Induces the Augmentation of Exocytotic Serotonin Release in Rat Organotypic Raphe Slice Cultures**Kazuki Nagayasu<sup>1</sup>, Maiko Kitaichi<sup>1</sup>, Hisashi Shirakawa<sup>1</sup>, Takayuki Nakagawa<sup>1,\*</sup>, and Shuji Kaneko<sup>1</sup><sup>1</sup>Department of Molecular Pharmacology, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501, Japan

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**Abstract.** 3,4-Methylenedioxymethamphetamine (MDMA) causes serotonin efflux via serotonin transporter. Recently, we have reported that sustained exposure to MDMA induced an augmentation of serotonin release in rat raphe serotonergic slice cultures. Here we investigated the mechanism of augmented serotonin release from the slice cultures. Sustained MDMA exposure had no effect on MDMA-induced serotonin efflux in the synaptosomal fraction, whereas either tetrodotoxin, calcium channel inhibitors, or AMPA-receptor antagonists significantly attenuated the augmented serotonin release. These results suggest that the increase in  $\text{Ca}^{2+}$ -dependent exocytotic serotonin release is mediated through activation of AMPA receptors and responsible for the sustained MDMA-induced augmentation of serotonin release.

**Keywords:** serotonin (5-HT), 3,4-methylenedioxymethamphetamine (MDMA), slice culture

3,4-Methylenedioxymethamphetamine (MDMA, “ecstasy”) is one of the widely abused recreational drugs in the world (1). Recreational drugs including MDMA are thought to be a significant public health problem due to its acute toxicity (2). Repeated administration of MDMA to rodents induces a progressive increase of locomotor activity known as behavioral sensitization (3), which is considered to represent a certain aspect of drug addiction (4). Several lines of evidence suggest the involvement of serotonergic neurons in behavioral responses to MDMA (5). Among various recognition sites, MDMA preferentially acts on serotonin transporter (SERT) and induces the carrier-mediated reverse transport of serotonin (5-HT) (efflux) via SERT (6). Indeed, in vivo microdialysis studies have shown that MDMA increases the 5-HT release in the striatum and prefrontal cortex, which is attenuated by a selective serotonin reuptake inhibitor (SSRI) but not by a voltage-dependent sodium channel inhibitor, tetrodotoxin (TTX) (7).

Recently, we have established rat organotypic raphe slice cultures that make serotonergic neurons applicable

to the analysis of acute and sustained effects of MDMA. Using the slice cultures, we found that acute treatment with MDMA increased 5-HT release and that sustained exposure to MDMA caused the augmentation of the MDMA-induced 5-HT release (8), although the molecular mechanisms remain to be clarified. In this study, we have examined the release mechanism of the augmented 5-HT release in the raphe slice cultures.

All animals were handled in accordance with the ethical guidelines of the Kyoto University Animal Research Committee and The Japanese Pharmacological Society. Organotypic raphe slice cultures were prepared as described previously (8), with slight modifications. Briefly, Wistar/ST rats at postnatal day 2 – 3 (Nihon SLC, Shizuoka) were anesthetized with hypothermia, decapitated, and the brain was isolated. Coronal sections (350- $\mu\text{m}$  thickness) containing the dorsal and median raphe nuclei were prepared with a tissue chopper (Narishige, Tokyo). Four slices were placed on each Millicell-CM insert (Millipore, Billerica, MA, USA). Slice cultures were maintained at the liquid/air interface for 14 – 16 days at 37°C in a 5%- $\text{CO}_2$  atmosphere. For measurement of 5-HT release, slices were preincubated in Krebs-Ringer-Henseleit (KRH) buffer (146 mM NaCl, 2.7 mM KCl, 1 mM  $\text{MgCl}_2$ , 1.2 mM  $\text{CaCl}_2$ , 10 mM D-glucose, 15 mM

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HEPES, 5 mM HEPES-Na, 0.2 mM ascorbic acid; pH 7.4) for 15 min and then transferred to fresh KRH buffer containing drugs and incubated for 30 min. After the incubation, KRH buffer was collected, and the extracellular 5-HT concentration was immediately analyzed with an HPLC/electrochemical detection system (Eicom, Kyoto).

For [ $^3\text{H}$ ]5-HT uptake and efflux assays, slice cultures were homogenized and centrifuged at  $1,000 \times g$ . The supernatants were centrifuged at  $18,000 \times g$ , and the resulting precipitates were resuspended in KRH buffer. The crude synaptosomal fraction was incubated in KRH buffer containing [ $^3\text{H}$ ]5-HT (5.22 TBq/mmol; GE Healthcare, Buckinghamshire, UK) at  $37^\circ\text{C}$  for 15 min. In the [ $^3\text{H}$ ]5-HT uptake assay, the incubation with [ $^3\text{H}$ ]5-HT ([total 5-HT] = 10 – 1000 nM) was terminated by rapid filtration, and the radioactivity was measured by a liquid scintillation counter. In the [ $^3\text{H}$ ]5-HT efflux assay, after preloading with [ $^3\text{H}$ ]5-HT ([total 5-HT] = 100 nM), the crude synaptosomal fraction was centrifuged at  $18,000 \times g$  again, and the precipitates were resuspended in KRH buffer. The synaptosomal fractions were transferred to fresh KRH buffer containing MDMA (1 – 1000  $\mu\text{M}$ ) and incubated for 30 min. Incubation was terminated by rapid filtration, and the amount of retained [ $^3\text{H}$ ]5-HT radioactivity in the synaptosomal fraction was measured. Non-specific binding was measured by the incubation on ice and was subtracted from the obtained counts. [ $^3\text{H}$ ]5-HT efflux was calculated by subtraction of retained [ $^3\text{H}$ ]5-HT radioactivity in the MDMA-treated synaptosomes from that in the vehicle-treated synaptosomes.

For Western blotting of SERT in the membrane fraction, slice cultures were homogenized and centrifuged at  $1,000 \times g$ . Supernatants were centrifuged at  $40,000 \times g$  for 20 min, and the resulting pellets were resuspended. Proteins were electrophoretically separated with polyacrylamide gel and transferred to a PVDF membrane. The membrane was probed with primary antibodies against SERT (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and  $\text{Na}^+/\text{K}^+$ -ATPase (1:5000; Abcam, Cambridge, MA, USA). Proteins were visualized by Immobilon Western HRP substrate (Millipore).

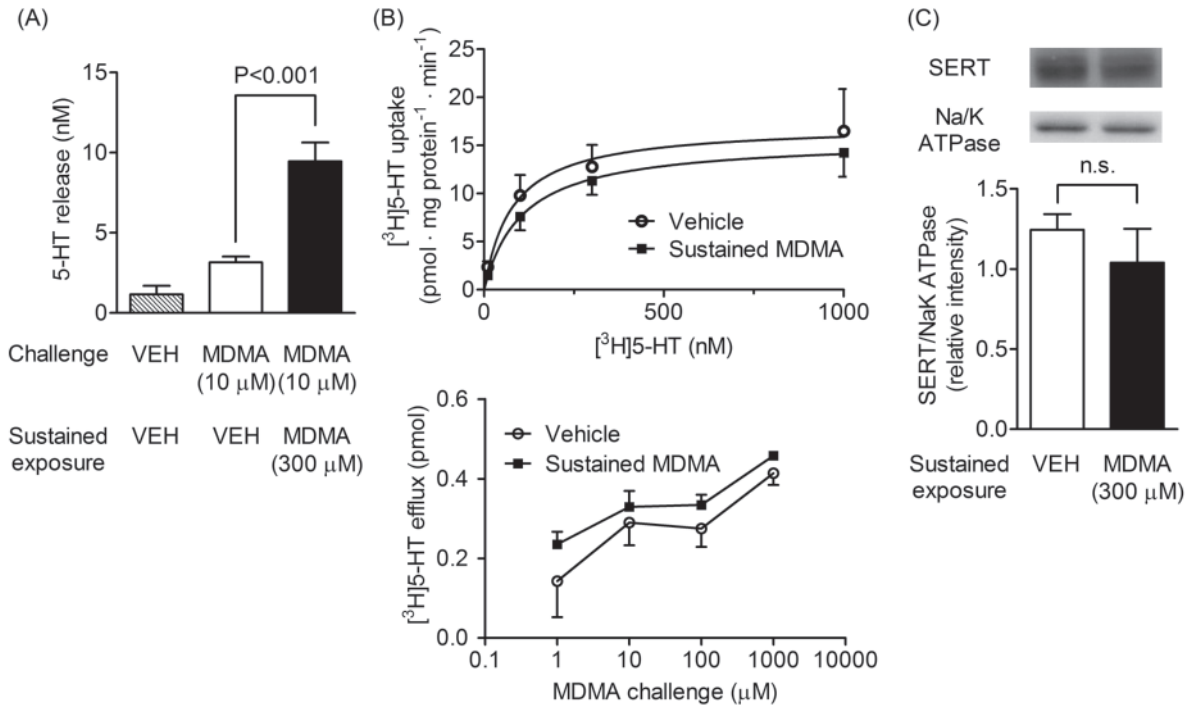
Data are presented as means  $\pm$  S.E.M. Differences were compared using Student's *t*-test or one-way ANOVA followed by the Bonferroni correction for multiple comparisons. In [ $^3\text{H}$ ]5-HT uptake and efflux assays, data were analyzed by two-way ANOVA. Differences with  $P < 0.05$  were considered significant.

After slice cultures were exposed to vehicle or MDMA (300  $\mu\text{M}$ ) in culture medium for 4 days, the slice cultures were transferred to fresh medium without MDMA and incubated for 1 day. Following the withdrawal period, slice cultures were challenged with MDMA (10  $\mu\text{M}$ ) in

KRH buffer for 30 min, and extracellular 5-HT concentration was measured ( $F_{2,14} = 28.82$ ,  $P < 0.001$ , Fig. 1A). As we have previously reported (8), the preexposure to MDMA caused significant augmentation of 5-HT release responding to the MDMA challenge, as compared to the control, vehicle-exposed slice cultures. To examine whether the augmented 5-HT release is due to the increase in MDMA-induced 5-HT efflux, we measured [ $^3\text{H}$ ]5-HT uptake and efflux using crude synaptosomes prepared from the slice cultures (Fig. 1B). In the [ $^3\text{H}$ ]5-HT uptake assay, there was no significant difference between the vehicle- and MDMA-exposed slice cultures in the 5-HT uptake into synaptosomes ( $F_{1,30} = 1.37$ ,  $P = 0.25$ , two-way ANOVA); the  $K_m$  values were  $78.9 \pm 55.5$  and  $106.9 \pm 50.6$  nM and the  $V_{\max}$  values were  $17.14 \pm 3.00$  and  $15.61 \pm 2.06$  pmol $\cdot\text{mg}$  protein $^{-1}\cdot\text{min}^{-1}$ , respectively. In the [ $^3\text{H}$ ]5-HT efflux assay, MDMA challenge (1 – 1000  $\mu\text{M}$ ) caused 5-HT efflux from synaptosomes in a concentration-dependent manner ( $F_{4,20} = 10.84$ ,  $P < 0.001$ , two-way ANOVA). However, there was no significant difference in MDMA-induced 5-HT efflux between vehicle- and MDMA-exposed slice cultures ( $F_{1,20} = 1.12$ ,  $P = 0.30$ , two-way ANOVA). Furthermore, we detected no significant change of SERT expression level in the membrane fraction prepared between from vehicle- and MDMA-exposed slice cultures (Fig. 1C). These results suggest that the MDMA exposure had no effect on the ability of SERT-mediated, acute 5-HT efflux.

Next, we investigated the contribution of exocytotic 5-HT release in the augmented 5-HT release, although it is considered to play a limited part in acute 5-HT release by MDMA (9). When vehicle-exposed slice cultures were challenged with MDMA, MDMA-induced 5-HT release was significantly inhibited by co-treatment with an SSRI, citalopram (0.3  $\mu\text{M}$ ), but not by TTX (1  $\mu\text{M}$ ) ( $F_{2,6} = 8.868$ ,  $P < 0.05$ ). In contrast, the augmented 5-HT release after MDMA exposure was significantly inhibited by co-treatment with TTX, but not by citalopram ( $F_{2,6} = 41.00$ ,  $P < 0.001$ , Fig. 2A). Furthermore, co-treatment with a specific P/Q-type voltage-dependent calcium channel (VDCC) inhibitor,  $\omega$ -agatoxin IVA (1  $\mu\text{M}$ ), significantly decreased the augmented 5-HT release in MDMA-exposed slice cultures, while it had no effect in sustained vehicle-exposed slice cultures (data not shown). However a specific N-type VDCC inhibitor,  $\omega$ -conotoxin MVIIA (1  $\mu\text{M}$ ), had no effect ( $F_{3,14} = 7.538$ ,  $P < 0.01$ , Fig. 2B). These results suggest that the augmented 5-HT release following sustained MDMA exposure is mediated through  $\text{Ca}^{2+}$ -dependent exocytotic release accompanied with action potential firings that cause opening of P/Q-type VDCC.

Since serotonergic neurons in the raphe nucleus are

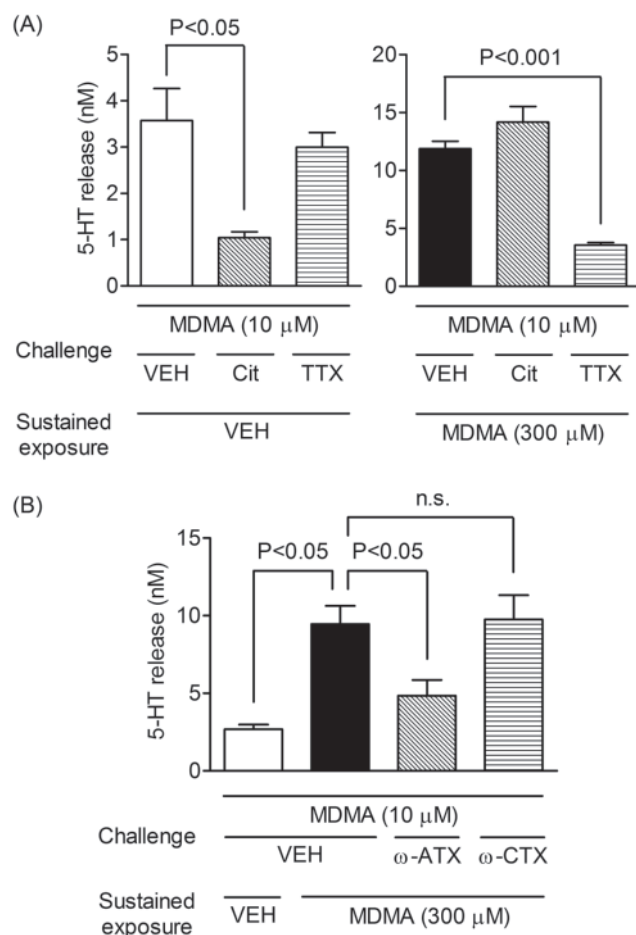


**Fig. 1.** 5-HT uptake and efflux rate in synaptosomal fraction and expression level of SERT in membrane fraction prepared from sustained MDMA-exposed slice cultures. A) Sustained MDMA-induced augmentation of 5-HT release. Slice cultures were exposed to vehicle (PBS) or MDMA (300  $\mu$ M) for 4 days. After a 1-day withdrawal period, slice cultures were challenged with MDMA (10  $\mu$ M), and 5-HT release was determined. Values represent means of 5-HT concentration  $\pm$  S.E.M.  $n = 5 - 6$ . B) 5-HT uptake and efflux rate in the synaptosomal fraction. In the crude synaptosomal fraction prepared from sustained vehicle- or sustained MDMA-exposed slice cultures following withdrawal period, [ $^3$ H]5-HT uptake into the synaptosomes (upper panel,  $n = 4 - 5$ ) and [ $^3$ H]5-HT efflux from the synaptosomes (lower panel,  $n = 3$ ) were determined. Values represent means  $\pm$  S.E.M. C) SERT expression in the membrane fraction. Upper panel shows representative blots for SERT (approximately 70 kDa) and Na<sup>+</sup>/K<sup>+</sup>-ATPase (approximately 100 kDa) as a control for sample loading. In the lower panel, values represent relative intensity of the SERT band normalized by the intensity of Na<sup>+</sup>/K<sup>+</sup>-ATPase  $\pm$  S.E.M.  $n = 3$ . n.s.: not significant, VEH: vehicle.

stimulated by AMPA/kainate receptors (10), we examined the effect of AMPA/kainate-receptor antagonists on the augmented 5-HT release after MDMA exposure. Co-treatment with an AMPA/kainate-receptor antagonist, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX, 30  $\mu$ M), inhibited the augmented 5-HT release in sustained MDMA-exposed slice cultures, whereas it had no effect on sustained vehicle-exposed slice cultures ( $F_{3,8} = 11.43$ ,  $P < 0.01$ , Fig. 3A). Similarly, a selective AMPA-receptor antagonist, GYKI52466 (30  $\mu$ M), significantly decreased the augmented 5-HT release, while a selective kainate-receptor antagonist, NS102 (10  $\mu$ M), had no effect ( $F_{3,31} = 10.63$ ,  $P < 0.001$ , Fig. 3B). These results suggest that the activation of AMPA receptors, but not kainate receptors, is responsible for the augmented 5-HT release induced by sustained MDMA exposure.

In this study, we have shown that the augmentation of 5-HT release after sustained exposure to MDMA is due to an increase in Ca<sup>2+</sup>-dependent exocytotic 5-HT release

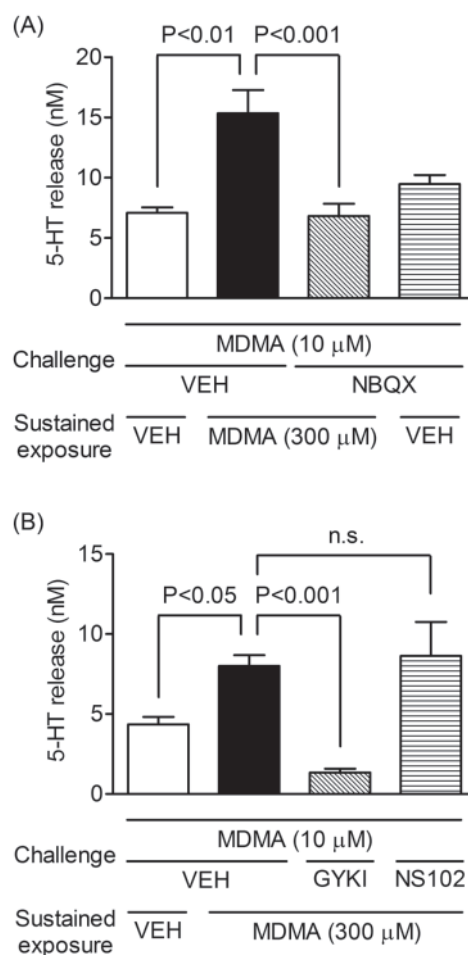
rather than the increase in 5-HT efflux via SERT, which is mediated through the activation of AMPA-type, but not kainate-type, glutamate receptors. Acute MDMA-induced 5-HT release is reported to be mainly due to 5-HT efflux via SERT (6, 7), while some reports indicate that acute MDMA induces, at least partly, exocytotic 5-HT release (11). Our present results, however, clearly indicate that acute MDMA-induced 5-HT release is due to 5-HT efflux, which was inhibited by an SSRI, being consistent with the observations in previous studies. Consistent with our study, *in vivo* studies have found that repeated administration of amphetamines causes augmentation of amphetamines-induced 5-HT release in the prefrontal cortex in parallel with behavioral sensitization (12, 13). In contrast, it was reported that repeated administration of MDMA according to a neurotoxic regimen induced no change or reduction in 5-HT release in the hippocampus or frontal cortex in response to electrical stimulation of the dorsal or median raphe nucleus (14). However, our present data suggest that sustained MDMA



**Fig. 2.** Effects of citalopram, tetrodotoxin, and VDCC inhibitors on the augmented 5-HT release. A) Slice cultures were exposed to vehicle (PBS) or MDMA (300  $\mu$ M) for 4 days. After a 1-day withdrawal period, slice cultures were challenged with MDMA (10  $\mu$ M) in the presence of citalopram (Cit: 0.3  $\mu$ M) or TTX (1  $\mu$ M), and 5-HT release was determined ( $n = 3$ ). B) After a 1-day withdrawal period, slice cultures were challenged with MDMA (10  $\mu$ M) in the presence of  $\omega$ -agatoxin IVA ( $\omega$ -ATX: 1  $\mu$ M) or  $\omega$ -conotoxin MVIIA ( $\omega$ -CTX: 1  $\mu$ M), and 5-HT release was determined ( $n = 3 - 6$ ). Values represent means of 5-HT concentration  $\pm$  S.E.M. n.s.: not significant, VEH: vehicle.

exposure could cause augmentation of exocytotic 5-HT release under a non-neurotoxic condition (8).

Several lines of evidence suggest that the glutamatergic system, especially AMPA/kainate glutamate receptors, is one of the important factors in drug addiction. The sensitized state induced by numerous types of drugs of abuse is mediated via increases in AMPA receptor responsiveness, which may occur through the induction, altered trafficking, and increased reactivity of AMPA receptors (15). The raphe serotonergic neurons are innervated by glutamatergic neurons, and co-treatment with AMPA and cyclothiazide, an inhibitor of AMPA receptor desensitization, is reported to induce 5-HT re-



**Fig. 3.** Effects of AMPA/kainate glutamate-receptor antagonists on the augmented 5-HT release. Slice cultures were exposed to vehicle (PBS) or MDMA (300  $\mu$ M) for 4 days. After a 1-day withdrawal period, slice cultures were challenged with MDMA (10  $\mu$ M) in the presence of vehicle (0.1% DMSO) or NBQX (30  $\mu$ M) (A), vehicle (0.1% DMSO), GYKI52466 (GYKI: 30  $\mu$ M) or NS102 (10  $\mu$ M) (B); and 5-HT release was determined. Values represent means of 5-HT concentrations  $\pm$  S.E.M.,  $n = 3$  (A) or  $n = 6 - 12$  (B). n.s.: not significant, VEH: vehicle.

lease in the dorsal raphe nucleus (10). Therefore, it is suggested that the activation of AMPA receptors increases the activity of the raphe serotonergic neurons, which causes sustained MDMA-induced augmentation of exocytotic 5-HT release. However, it is unclear whether activation of P/Q-type VDCC caused 5-HT release at the serotonergic terminals or glutamate release from the glutamatergic terminals. The increased glutamate may contribute to 5-HT release via activation of AMPA receptors, although we observed no detectable difference in glutamate release during MDMA challenge between sustained vehicle- and sustained MDMA-treated slice cultures (our preliminary data). To elucidate

the mechanisms underlying MDMA-induced augmentation of 5-HT release, further investigations will be needed.

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