

Enhancement of the Serotonin-Mediated Acetylcholine Release by Repeated Desmethylimipramine Treatment in the Hippocampus of Freely Moving Rats

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ABSTRACT—A possible involvement of serotonin-mediated cholinergic activation in the antidepressant effect of desmethylimipramine (DMI) was investigated by determination of the effects of a single or repeated DMI administration on acetylcholine (ACh) release in the hippocampus using an *in vivo* microdialysis technique and a radioimmunoassay for ACh. Rats were administered DMI (10 mg/kg, *i.p.*) acutely or repeatedly for 21 days. A single or repeated DMI administration did not cause any significant effects on the basal ACh release compared with the respective controls. Atropine perfusion in the acutely DMI-treated or control rats increased the ACh release to the same degree. In repeatedly DMI-treated rats, serotonin (5-HT) (1 to 10 μ M) perfusion enhanced significantly the ACh release. However, 5-HT in acutely DMI-treated rats enhanced significantly the ACh release only at 10 μ M. 5-HT did not cause any changes in ACh release in control rats. Hippocampal 5-HT content of acutely DMI-treated rats was significantly higher than that of saline-treated control rats, while no difference was observed between the repeatedly DMI- and saline-treated rats. These findings suggest, for the first time, that DMI induced a facilitation of cholinergic neurotransmission in the rat hippocampus through the activation of 5-HT-receptor function.

Keywords: Acetylcholine, Desmethylimipramine, Hippocampus, Microdialysis, Serotonin (5-HT)

Tricyclic antidepressants such as imipramine and desmethylimipramine (DMI) are known to potentiate the action of biogenic amines in the central nervous system by blockade of reuptake at nerve terminals. DMI is one of the most potent of the group in blocking noradrenaline reuptake, but it is 100- to 1000-fold less potent as an inhibitor of serotonin (5-HT) reuptake (1). It is generally accepted that about 2 to 3 weeks must pass before the therapeutic effects of DMI are evident, while the blocking effect on the reuptake can develop promptly (2). Thus, there is increasing doubt that inhibition of the reuptake of noradrenaline or 5-HT *per se* is a sufficient explanation for the antidepressant action of these drugs. In addition, some newly developed antidepressants such as mianserin may not inhibit reuptake of biogenic amines, although they may raise synaptic biogenic amine levels by other mechanisms (3).

The hippocampus receives cholinergic, serotonergic and noradrenergic projections from the septum, median

raphe nucleus and locus coeruleus, respectively (4). Some electrophysiological *in vivo* and *ex vivo* studies have shown that repeated treatment of rats with tricyclic antidepressants caused an enhanced responsiveness to 5-HT by increasing the sensitivity of post-synaptic 5-HT_{1A} receptors in the rat hippocampus (5–7). The role of the 5-HT_{1A} receptor subtype has been extensively investigated using a potent and selective 5-HT_{1A}-receptor agonist, 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), and several partial agonists such as buspirone and ipsapirone (8, 9), because of the potential use of the 5-HT_{1A} receptor as a therapeutic target in the treatment of affective disorders, including depression and anxiety. Thus, involvement of changes in central serotonergic transmission have long been considered in the mechanism of the therapeutic action of tricyclic antidepressants. However, the interactions among the cholinergic, serotonergic and noradrenergic nervous systems were not fully investigated in terms of the mechanisms involved in the therapeutic action of tricyclic antidepressants. Several groups of investigators have recently reported the involvement of

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5-HT, at least partly via 5-HT_{1A} receptors, in the regulation of acetylcholine (ACh) release in the hippocampus (10–16). Since interactions among cholinergic, serotonergic and noradrenergic systems exist in the hippocampus, long-term administration of DMI may modulate functional coupling among these systems.

Surgical or pharmacological manipulations of the ascending brainstem monoaminergic systems, which innervate wide areas of the forebrain, including the septum and the hippocampal formation, have been shown to have marked effects on septo-hippocampal cholinergic activity (17). In addition, repeated administration of DMI has been reported to cause β -adrenergic desensitization, which involves both a rapid attenuation of adenylyl cyclase responsiveness and a gradual down-regulation of receptor number in the rat brain (18). Therefore, treatment with DMI may produce some effects on the hippocampal ACh release through the changes of β -adrenergic mechanisms.

In view of the above findings, we attempted to investigate the effect of acutely or repeatedly administered DMI on the cholinergic neurotransmission in the hippocampus of freely moving rats using the *in vivo* microdialysis technique and a radioimmunoassay (RIA) for the determination of ACh. We also studied whether treatment with DMI alters the hippocampal cholinergic neurotransmission via serotonergic and β -adrenergic mechanisms by local application of 5-HT or isoproterenol, a non-selective β -adrenoceptor agonist, through the microdialysis probe.

MATERIALS AND METHODS

Chemicals and apparatus

ACh iodide, atropine sulfate and physostigmine sulfate were purchased from Wako Pure Chemicals (Osaka). DMI hydrochloride, 5-HT creatinine sulfate and isoproterenol hydrochloride were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of reagent grade and obtained from commercial sources.

The composition of the Ringer solution used as a perfusion medium was 147 mM NaCl, 4 mM KCl and 3.4 mM CaCl₂ (19). Physostigmine (10 μ M) was added to Ringer solution. DMI chloride was dissolved in physiological saline at concentrations that allowed the required dose to be administered *i.p.* in a volume of 2 ml/kg. For local application into the hippocampus through the microdialysis probe, 5-HT creatinine sulfate, isoproterenol hydrochloride and atropine sulfate were dissolved in Ringer solution.

Microdialysis probes (CMA/12) with an outer diameter of 0.5 mm, a length of 3 mm, and a molecular weight cut-off of 20,000 Da were obtained from CMA

/Microdialysis AB (Stockholm, Sweden). The average *in vitro* recovery ratio of ACh from the microdialysis probe placed in a standard ACh solution at a concentration of 30 ng/ml was $18.1 \pm 0.3\%$ ($n=104$). *In vivo* values shown in the text and figures were not corrected for *in vitro* recovery.

Surgery for microdialysis

Male Wistar rats weighing 250–320 g were obtained from Sankyo Labo Service Corporation (Tokyo) and housed under a 12-hr (8:00–20:00) light / 12-hr (20:00–8:00) dark cycle with food and water available *ad libitum* for at least one week before the experiment. The surgical procedure used has been described previously elsewhere (20). Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg, *i.p.*) and placed in a stereotaxic frame (SR-6, SM-15/S; Narishige, Tokyo) with an incisor bar. The skull was exposed and a hole was drilled for implantation of a microdialysis probe into the hippocampus at an angle of 10 degrees from the vertical as described previously (20). The coordinates of the dialysis probe tip were A 3.80, L 5.20 and V 1.60, according to the rat brain stereotaxic atlas of Paxinos and Watson (21). The microdialysis probe was kept in place by skull screws and acrylic dental cement. The rats were allowed to recover from the surgery for two days before the microdialysis experiment. This interval is known to be sufficient for cessation of bleeding and restoration of blood flow and metabolism in the area around the probe (22).

Microdialysis experiments

Either DMI at a dose of 10 mg/kg or saline was administered *i.p.* once daily for 21 days. Microdialysis experiments were performed in conscious, freely moving rats either 2.5 hr after the single or 24 hr after repeated administration of DMI or saline for 21 days. The microdialysis probe was perfused with Ringer solution containing 10 μ M physostigmine using a pump (EP-60; Eicom, Kyoto) at a rate of 2 μ l/min. The perfusate was discarded for the first 60 min and then collected at 15-min intervals as shown in each figure. The perfusates were collected into tubes containing 120 μ l of 0.01 M acetic acid and left to stand in an ice-water bath.

Atropine, 5-HT and isoproterenol, dissolved in the perfusion fluid at the concentrations shown in the figures, were applied locally to the hippocampus via the microdialysis probe. The concentration of these solutions was raised serially at 60-min intervals.

The amount of ACh in the perfusate was determined by RIA in duplicate as described below, using a 50- μ l portion of the content of each tube.

Procedures for determination of ACh by RIA

The extracellular levels of ACh were estimated by measuring the ACh content of the perfusate by RIA using rabbit antiserum raised against choline hemiglutarate-bovine serum albumin conjugates and [3 H]ACh (23). This RIA is specific for ACh and its levels of cross-reactivity with choline, phosphatidylcholine, and phosphorylcholine are less than 0.012% (23, 24). The limit of sensitivity is about 20 fmol (3 pg)/tube (20, 25). Detailed procedures for determination of ACh in the microdialysis perfusate by RIA were described elsewhere (11, 15, 20, 25, 26). Briefly, for the determination of the ACh content of the perfusate, a 50- μ l portion of the collected sample was added to an assay tube containing 100 μ l of antiserum (1:700) diluted in 0.4% gamma-globulin solution, 250 μ l of 0.15 M Tris-HCl buffer, and 100 μ l of [3 H]ACh (about 5000 dpm). The tubes were then incubated overnight at 4°C. The antibody-bound [3 H]ACh was then separated by an ammonium sulfate method (27), and the radioactivity of the antibody-bound fraction was counted in a liquid scintillation counter. To correct for substances other than ACh that could influence the RIA, tubes containing volumes of unused perfusion fluid similar to those tubes used for the RIA of ACh served as blanks. The amount of ACh in the collected perfusate was calculated by subtracting the value for the blank from that for the unknown sample.

Determination of 5-HT in the hippocampus

After the end of the microdialysis experiments, the content of 5-HT in the hippocampus of 6 rats treated with a single or repeated DMI was determined by the method of Mead and Finger (28) as previously described in detail (15).

Statistics

The content of ACh in the perfusate was expressed either as picograms of ACh per 15-min sample (Table 1 and Fig. 1) or as a percentage of the baseline value (the average amount of ACh in three-sample collections immediately preceding the test treatment) (Figs. 2 and 3).

The values shown in the tables and figures are means \pm S.E.M. Student's *t*-test was used for comparison of the data between the saline- and DMI-treatment groups (Tables 1 and 2). The significance of the differences between drug and vehicle treatment conditions and between drug and vehicle perfusion conditions was determined by repeated measures analysis of variance (ANOVA) for factorial design after logarithmic transformation of the response values to keep variance homogeneity. When significance was indicated, the differences were assessed by the post hoc test for least squares means between groups. In addition, for exploratory analyses,

Student's *t*-tests were used for comparison of the data between the saline and DMI treatment groups, and Dunnett's tests were used for comparison of the data observed at 0 min with those at different time points in each group and for comparison of the data observed between the perfusion of Ringer solution and 5-HT or isoproterenol perfusion at each time point. Differences at $P < 0.05$ were considered significant.

RESULTS

Effect of acute and repeated DMI treatment on basal ACh release in the hippocampus

The baseline values of ACh content in the hippocampal perfusate (the average amount of ACh in three-sample collections immediately preceding the test treatment) in saline- or DMI-treated rats used in the present study are summarized in Table 1. Both acute (Figs. 1 and 2B, $n=28$) and repeated DMI treatment (Fig. 3B, $n=23$) did not cause any significant effects on the basal ACh release compared with the respective controls (acute saline treatment, Figs. 1 and 2A, $n=28$; repeated saline treatment, Fig. 3A, $n=25$).

Effects of atropine perfusion on the ACh release in the hippocampus of acutely DMI-treated rats

ACh release in the brain is regulated by a negative feedback mechanism through presynaptic muscarinic autoreceptors (25, 29–32). In order to investigate whether a weak antimuscarinic effect of DMI (33–35) affects the basal ACh release in DMI-treated rats, we examined the effect of local application of atropine on ACh release 2.5 hr after a single administration of DMI. Atropine perfusion caused a dose-dependent and significant increase of the ACh release in the hippocampus of rats administered either saline or DMI (Fig. 1) ($F(12, 120)=25.24$; $P=0.0001$, repeated measures ANOVA). However, no difference in the increase rate of the ACh release was

Table 1. Effect of a single and repeated DMI treatment on the basal ACh release in the hippocampus

Treatment	Basal ACh content in the hippocampal perfusate (pg/15 min)
After a single i.p. administration	
saline ($n=28$)	209.7 \pm 21.6
DMI (10 mg/kg) ($n=28$)	241.3 \pm 23.8
After repeated i.p. administration for 21 days	
saline ($n=25$)	153.2 \pm 17.9
DMI (10 mg/kg per day) ($n=23$)	176.5 \pm 23.8

Each value represents a mean \pm S.E.M.

observed between the saline- and DMI-treatment ($F(12, 120)=0.88$; $P=0.573$, repeated measures ANOVA).

Effects of 5-HT or isoproterenol perfusion on the ACh release in the hippocampus of acutely DMI-treated rats

Perfusion of the dialysis probe with Ringer solution in the hippocampus demonstrated a stable amount of ACh release for 3.5 hr in both acutely saline- (Fig. 2A) and DMI-treated rats (Fig. 2B).

Local application of 5-HT ($1-10 \mu\text{M}$) and isoproterenol ($0.1-1 \mu\text{M}$) to the hippocampus of acutely saline-treated rats through the dialysis probe did not produce any change in the ACh release in the hippocampus (Fig. 2A).

Perfusion of the hippocampus with various drug solutions caused a significant effect on the ACh efflux in acutely DMI-treated rats ($F(2, 38)=3.84$; $P=0.0304$, repeated measures ANOVA) (Fig. 2B). The post hoc test revealed that 5-HT perfusion significantly increased the ACh efflux relative to the perfusion of the Ringer solution and isoproterenol ($P=0.0019$ and $P=0.0050$, respectively), while no significant differences were observed among 5-HT, Ringer solution and isoproterenol perfusion in the saline-treated rats. The ACh efflux following 5-HT perfusion was significantly increased at 120 and 150 min when compared with the value observed at 0 min in DMI-treated rats ($P<0.05$ by Dunnett's test). Isoproterenol did not cause any significant changes in the ACh release in the hippocampus compared with the data observed at 0 min.

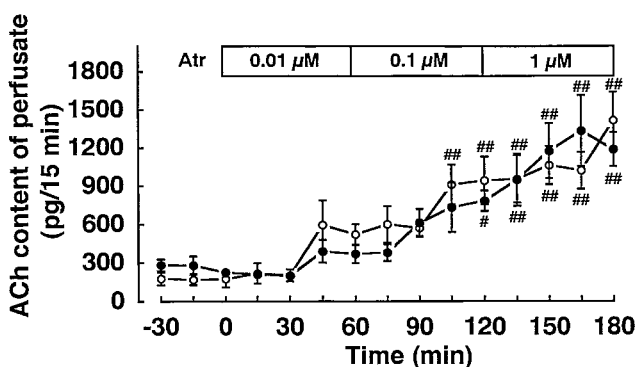


Fig. 1. Effect of atropine on the basal acetylcholine (ACh) release in the hippocampus of rats treated with a single i.p. dose of saline or DMI (10 mg/kg). Either saline (\circ) or DMI (\bullet) was administered 2.5 hr before the onset of atropine perfusion. Each point represents the mean \pm S.E.M for 6 rats. Atropine perfusion caused a dose-dependent and significant increase of the ACh release in the hippocampus of rats administered either saline or DMI ($F(12, 120)=25.24$; $P=0.0001$, repeated measures ANOVA). $^{\#}P<0.05$, $^{##}P<0.01$, compared with the value at 0 min (Dunnett's test). No significant difference was detected between the treatments by repeated measures ANOVA.

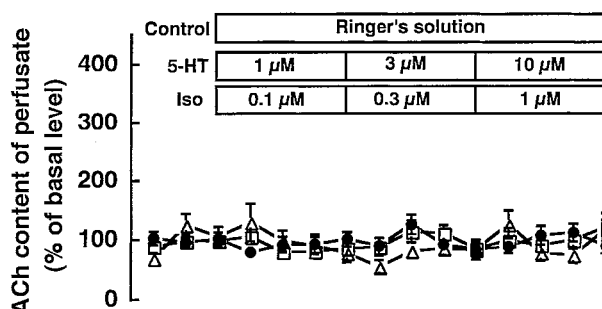
Effects of 5-HT or isoproterenol perfusion on the ACh release in the hippocampus of repeatedly DMI-treated rats

Perfusion of the dialysis probe with Ringer solution in the hippocampus caused a stable amount of ACh release for 3.5 hr in both repeatedly saline- (Fig. 3A) and repeatedly DMI-treated rats (Fig. 3B).

Local application of Ringer solution alone, 5-HT and isoproterenol to the hippocampus of repeatedly saline-treated rats through the dialysis probe did not produce any change in the ACh release in the hippocampus (Fig. 3A).

Perfusion of the hippocampus with various drug solutions caused a significant effect on the ACh efflux in

A Saline



B Desmethylinipramine

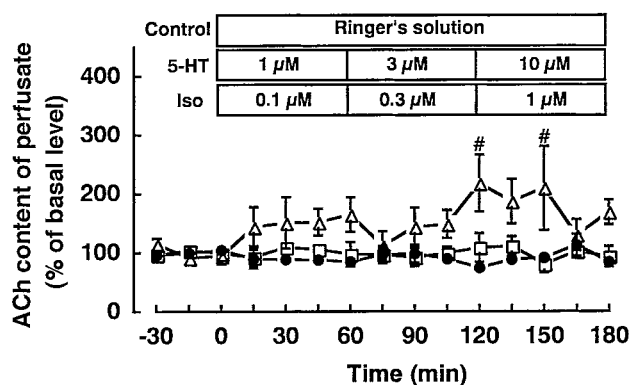


Fig. 2. Effects of local application of 5-HT and isoproterenol on acetylcholine (ACh) release in the hippocampus of rats treated with a single i.p. dose of saline (A) or DMI (10 mg/kg) (B). Either 5-HT (Δ), isoproterenol (\square) or Ringer solution (\bullet) was perfused into the hippocampus during the time indicated by the bar. Each point represents the mean \pm S.E.M for 6 to 8 rats. Perfusion of various drug solutions caused a significant effect on the ACh efflux in DMI-treated rats ($F(2, 38)=3.84$; $P=0.0304$, repeated measures ANOVA) (B). Post hoc tests for least squares means revealed that 5-HT perfusion significantly increased the ACh efflux relative to the Ringer solution and isoproterenol in DMI-treated rats ($P=0.0019$ and $P=0.0050$, respectively). $^{\#}P<0.05$, compared with the value at 0 min (Dunnett's test).

repeatedly DMI-treated rats ($F(2, 42)=13.99$; $P=0.0001$, repeated measures ANOVA) (Fig. 3B). Post hoc tests for least squares means revealed that 5-HT perfusion significantly increased the ACh efflux relative to the perfusion of the Ringer solution and isoproterenol in repeatedly DMI-treated rats ($P=0.0001$ and 0.0001 , respectively), while no significant differences were observed among 5-HT, Ringer solution and isoproterenol perfusion in saline-treated rats. The ACh efflux following 5-HT perfusion was significantly increased when compared with the perfusion of Ringer solution at 30, 45, 60, 75, 90, 120, 135, 150, 165 and 180 min ($P<0.05$ or $P<0.01$ by Dunnett's test); and it was significantly increased at 60, 120, 150, 165 and 180 min when compared with the value

observed at 0 min ($P<0.05$ or $P<0.01$ by Dunnett's test). Isoproterenol did not cause any significant changes in the ACh release in the hippocampus compared with the data observed at 0 min.

Effect of acute and repeated DMI treatment on the 5-HT content in the hippocampus

A significant increase of the 5-HT content in the hippocampus of acutely DMI-treated rats was observed when compared with that in the acutely saline treated rats ($P<0.01$ by Student's *t*-test) (Table 2). However, after repeated treatment, no difference in the 5-HT content in the hippocampus was observed between saline and DMI.

DISCUSSION

Antimuscarinic agents such as atropine and scopolamine increase ACh release in the brain through the inhibition of presynaptic muscarinic autoreceptors when a cholinesterase inhibitor is included in the perfusion fluid in the *in vivo* microdialysis experiments (25, 29–32). Although DMI has been reported to have a weak antimuscarinic activity (33–35), DMI at a single or repeated *i.p.* dose of 10 mg/kg caused no significant effects on the basal ACh release. In addition, we investigated whether DMI potentiates the ACh release caused by an antimuscarinic agent, atropine. However, the increase rate of ACh release induced by atropine after a single dose of DMI was virtually the same as that after a single dose of saline, suggesting that the antimuscarinic action of DMI under the present experimental conditions is not large enough to affect the basal ACh release.

The data presented in this paper show, for the first time, that DMI treatment induces an enhancement of central cholinergic neurotransmission through a 5-HT-mediated pathway. 5-HT perfusion in the hippocampus increased the ACh release significantly in both acutely

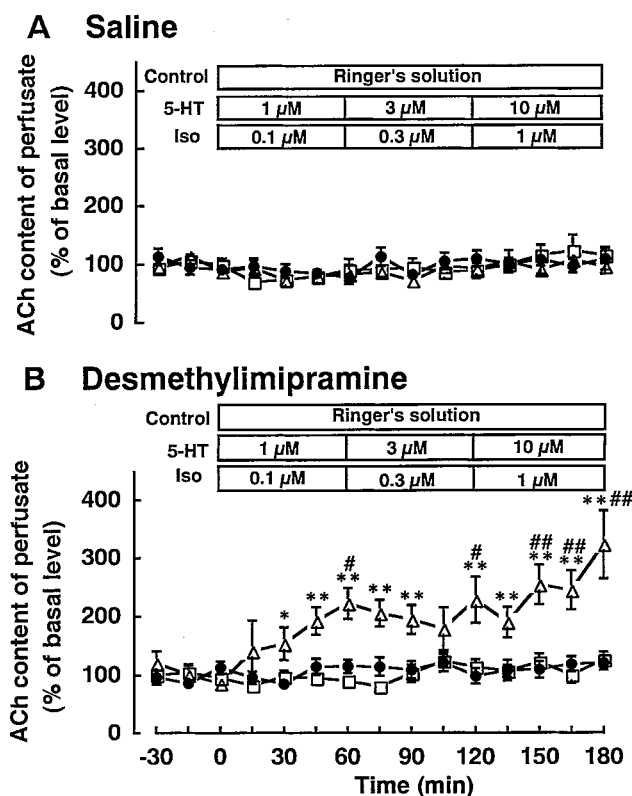


Fig. 3. Effects of local application of 5-HT and isoproterenol on acetylcholine (ACh) release in the hippocampus of rats treated with repeated *i.p.* doses of saline (A) or DMI (10 mg/kg) for 21 days (B). Either 5-HT (Δ), isoproterenol (\square) or Ringer solution (\bullet) was perfused into the hippocampus during the time indicated by the bar. Each point represents the mean \pm S.E.M for 6 to 9 rats. Perfusion of various drug solutions caused a significant efflux on the ACh efflux in repeatedly DMI-treated rats ($F(2, 42)=13.99$; $P=0.0001$, repeated measures ANOVA) (B). Post hoc tests for least square means revealed that 5-HT perfusion significantly increased the ACh efflux relative to the perfusion of the Ringer solution and isoproterenol in repeatedly DMI-treated rats ($P=0.0001$ and $P=0.0001$, respectively). * $P<0.05$, ** $P<0.01$, compared with the Ringer solution (Dunnett's test). # $P<0.05$, ## $P<0.01$, compared with the value at 0 min (Dunnett's test).

Table 2. Effect of a single and repeated DMI treatment on the hippocampal 5-HT content

Treatment	5-HT content (μ g/g tissue)
After a single <i>i.p.</i> administration	
saline (n=6)	5.57 \pm 0.21
DMI (10 mg/kg) (n=6)	7.51 \pm 0.43**
After repeated <i>i.p.</i> administration for 21 days	
saline (n=6)	6.19 \pm 0.24
DMI (10 mg/kg per day) (n=6)	6.56 \pm 0.24

Each value represents a mean \pm S.E.M. ** $P<0.01$, compared with the saline-treated control (one-way of ANOVA followed by Student's *t*-test).

and repeatedly DMI-treated rats, while it did not produce any changes of ACh release in the saline-treated control rats. The enhancement of ACh release by 5-HT was more pronounced and consistent in repeatedly DMI-treated rats than that in acutely DMI-treated rats. The content of 5-HT in the hippocampus was increased significantly by the acute DMI treatment, while no difference was observed after repeated DMI treatment. Therefore, an acute elevation of 5-HT concentration at the receptor sites in the hippocampus itself does not appear to contribute much to the increase of ACh release induced by DMI. An augmentation of the sensitivity of 5-HT receptor function, especially 5-HT_{1A} receptors in the hippocampus, induced by repeated DMI treatment (5–7) may be related to the facilitation of cholinergic neurotransmission observed in the present study. The data from our laboratory (11, 15) and others (12) showed a potentiation of ACh release from the rat hippocampus by locally applied 8-OH-DPAT, a 5-HT_{1A} agonist, in the presence of physostigmine in the perfusion fluid, suggesting that the stimulation of 5-HT_{1A} receptors in the hippocampus induces facilitation of cholinergic neurotransmission under certain conditions. Taken together, these findings further support the possibility that the enhancement of 5-HT-induced ACh release by DMI is ascribed, at least in part, to changes in the affinity of 5-HT for 5-HT_{1A} receptors or in its function.

Although the relationship between the antidepressant effect of DMI and the enhancement of basal or 5-HT-induced ACh release in the hippocampus remains to be elucidated, cholinergic neurotransmission is of particular importance in the mechanisms of attention as well as learning and memory. It has recently been shown that 5-HT_{1A} agonists such as buspirone have anxiolytic and antidepressant properties (36) and that 5-HT_{1A}-receptor antagonists such as NAN 190 or BMY 7378 block the antidepressant-like effects of DMI or 8-OH-DPAT, suggesting an involvement of 5-HT_{1A}-receptor activation in the antidepressant effects of DMI (37). In addition, Nakai et al. (15) have suggested that hippocampal postsynaptic 5-HT_{1A} stimulation is involved mainly in locally applied 8-OH-DPAT-induced increase of ACh release. Taken together, these findings suggest that the 5-HT-induced increase of hippocampal ACh release in repeatedly DMI-treated rats appears to be mediated through the postsynaptic 5-HT_{1A} receptors and that an enhancement of cholinergic neurotransmission in the hippocampus is related, at least in part, to the mechanism of antidepressant action of DMI.

DMI causes β -adrenergic desensitization in the rat brain (18). However, we did not observe any effect of isoproterenol perfusion into the hippocampus on the ACh release in both saline-treated control and acutely or

repeatedly DMI-treated rats in the present study. These results suggest that ACh release in the rat hippocampus may not be regulated at least via β -adrenoceptors.

In conclusion, repeated administration of DMI enhances cholinergic neurotransmission via 5-HT receptors, but not via β -adrenoceptors, in the hippocampus of freely moving rats.

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