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Effects of L-type calcium channel antagonists on the serotonin-depleting actions of MDMA in rats

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The calcium channel antagonists verapamil nifedipine and flunarizine all increased the threshold for convulsions induced by *N*-methyl-D-aspartate in rats. By contrast, only flunarizine blocked the long-term serotonin-depleting effects of 3,4-methylenedioxymethamphetamine. Flunarizine was also the only drug that antagonized methamphetamine-induced stereotypy. These findings suggest that calcium influx through L-type channels does not participate in the neurotoxic mechanism of MDMA, and that the neuroprotective actions of flunarizine are probably related to its anti-dopaminergic activity.

Amphetamine and several of its structural analogs have been shown to damage aminergic nerve terminals in the CNS of rodents and non-human primates by an uncertain mechanism[21]. Recent studies demonstrating that the neurotoxic effects of the amphetamines can be prevented by *N*-methyl-D-aspartate (NMDA) antagonists have, however, focused attention on the possibility that glutamatergic systems may play an important role in the toxic mechanism of action of these stimulant drugs[6,7,22,23]. This is an attractive possibility given the extensive evidence linking alterations in glutamatergic neurotransmission with the neuronal-damaging effects associated with several types of CNS injury, such as trauma, hypoxemia or hypoglycemia[1].

Many of the effects of glutamate are thought to be related to an influx of extracellular calcium. While evidence suggests that an activation of NMDA receptor-gated calcium channels provides an important avenue for this influx of calcium[1], calcium entry via voltage-sensitive channels also contribute to the rise in cytosolic calcium because of the depolarizing actions of glutamate[3]. The contribution of the voltage-sensitive channel to the increase in cytosolic calcium appears to be important in at least some situations because L-type calcium channel antagonists (CCA's), like NMDA antagonists, have been reported to prevent the neuronal-damaging effects of hypoxemia/ischemia under certain conditions[18,20,27]. Similarly, both L-type CCA's and NMDA antagonists prevent the convulsive effects of intravenously administered NMDA in mice[11]. Voltage-sensitive calcium channels may also play a role in the neurotoxic effects of the amphetamines as the CCA flunarizine attenuates the toxic effects of 3,4-methylenedioxymethamphetamine (MDMA) on serotonergic neurons[10,15]. Based on these findings, we investigated the involvement of voltage-sensitive calcium channels in the neurotoxic effects of MDMA by determining the ability of different L-type CCA's to block the long-term serotonin-depleting effects of the neurotoxin in rats. The results indicate that although flunarizine blocks the long-term serotonin-depleting effects of MDMA, nifedipine and verapamil are ineffective.

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The data suggest that calcium influx through L-type channels does not participate in the mechanism by which MDMA damages serotonergic neurons.

Male Sprague–Dawley rats, weighing 180–200 g, were used. Haloperidol, nifedipine and verapamil were obtained from the Sigma Chemical Company (St. Louis, MO), while MDMA was obtained from the National Institute on Drug Abuse. Flunarizine was a gift from Janssen Pharmaceuticals (Piscataway, NJ). All drugs were prepared in sterile 0.9% saline solution immediately prior to injection; flunarizine, haloperidol and nifedipine were suspended with the aid of 1% Tween and ultrasound. Drug doses are expressed as the salt, with the exception of MDMA which is expressed as the free base.

Recent studies indicate that the capacity of NMDA to produce convulsions in mice can be prevented by nifedipine, verapamil or diltiazam, an effect attributed to the shared ability of these agents to block neuronal L-type voltage-sensitive calcium channels[11]. These experiments were repeated here in rats, in order to establish a basis for the doses of the CCA's to be tested in the neurotoxicity experiments. Groups of rats were pretreated i.p. with single doses of either saline, nifedipine (20, 25 or 30 mg/kg), verapamil (5, 10 or 20 mg/kg) or flunarizine (15, 20 or 30 mg/kg) 30 min before the i.v. (tail vein) constant infusion of racemic NMDA (NMDLA). Rats were infused with NMDLA (20 mg/ml; 0.54 ml/min) until a minimal convulsion (forelimb clonus) was observed[12].

For the neurotoxicity experiments, groups of rats were treated s.c. four times with either saline or MDMA alone (7.5 mg/kg per injection, each injection separated by an interval of 2 h)[7]. Separate groups were treated i.p. three times with nifedipine (30 mg/kg per injection) or verapamil (25 or 50 mg/kg per injection), either alone or concurrently with MDMA. Groups given the concurrent drug treatments were injected with nifedipine or verapamil 30 min before the first, second and fourth dose of MDMA. In other experiments, groups of rats were injected i.p. four times with flunarizine (15 or 20 mg/kg/injection) or haloperidol (1 mg/kg per injection), either alone or concurrently with MDMA. Rats receiving concurrent drug treatments were injected with flunarizine or haloperidol 30 min before each of the four doses of MDMA. Doses of the CCA's were selected based on their ability to significantly elevate the NMDA convulsive threshold. A multiple-dosing regimen for the CCA's was employed in order to maximize the possibility that protection would be observed. All animals were killed 1 week after the completion of their drug-dosing regimen by decapitation, and the concentrations of serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) determined in the hippocampus and frontal cortex, as described elsewhere[7].

The ability of flunarizine or verapamil to inhibit the stereotypic behavioral effects of methamphetamine was also investigated. Here, rats were pretreated i.p. with single doses of either saline, flunarizine (5, 10, 20 mg/kg) or verapamil (20 mg/kg) 30 min before the i.p. administration of methamphetamine (6 mg/kg). The presence or absence of stereotypy was assessed visually 30 min later in individual rats, as described elsewhere[12].

Consistent with previous studies in mice[11], all three of the CCA's were found to elevate the NMDLA

TABLE I

Effects of flunarizine and haloperidol on the serotonin-depleting effects of MDMA

Rats (8 per group) were killed 1 week after the completion of the drug-dosing regimen for the assay of serotonin and 5-HIAA (5-HIAA data not shown). Data are from two separate experiments and are presented as the mean ± SD, or as a percentage of control. Differences between individual groups were compared posthoc by Student's *t*-test (corrected for multiple comparisons by the method of Bonferroni) after a one-way analysis of variance showed a statistically significant difference among groups (flunarizine experiment – hippocampus: $F = 14.2$, $P < 0.0001$; cortex: $F = 15.2$, $P < 0.0001$. Haloperidol experiment – hippocampus: $F = 18.1$, $P < 0.0001$; cortex: $F = 7.2$, $P < 0.0002$).

Pretreatment	*Treatment*	*Hippocampus (ng / mg tissue)*	*Cortex (ng / mg tissue)*
Saline	saline	0.30 ± 0.03	0.16 ± 0.03
Flunarizine (20 mg/kg)	saline	0.29 ± 0.01 (97%)	0.19 ± 0.02 (118%)
Saline	MDMA	0.15 ± 0.06 (50%) **	0.07 ± 0.03 (44%) **
Flunarizine (15 mg/kg)	MDMA	0.24 ± 0.04 (80%) ††	0.14 ± 0.03 (87%) ††
Flunarizine (20 mg/kg)	MDMA	0.30 ± 0.05 (100%) ††	0.16 ± 0.03 (100%) ††
Saline	saline	0.38 ± 0.04	0.26 ± 0.06
Haloperidol (1 mg/kg)	saline	0.42 ± 0.01 (111%)	0.26 ± 0.02 (100%)
Saline	MDMA	0.22 ± 0.05 (58%) **	0.16 ± 0.08 (61%) *
Haloperidol (1 mg/kg)	MDMA	0.36 ± 0.03 (95%) ††	0.22 ± 0.02 (85%)

* $P < 0.05$, ** $P < 0.01$, compared to saline control.
† $P < 0.05$, †† $P < 0.01$, compared to MDMA alone.

TABLE II

Effects of verapamil and nifedipine on the serotonin-depleting effects of MDMA

Rats (6–14 per group) were killed 1 week after the completion of the drug-dosing regimen for the assay of serotonin and 5-HIAA (5-HIAA data not shown). Data are presented as the mean ± SD, or as a percentage of control. Differences between individual groups were compared posthoc by Student's *t*-test (corrected for multiple comparisons by the method of Bonferroni) after a one-way analysis of variance showed a statistically significant difference among groups (hippocampus: $F = 27.4$, $P < 0.0001$; cortex: $F = 21.7$, $P < 0.0001$).

Pretreatment	*Treatment*	*Hippocampus (ng / mg tissue)*	*Cortex (ng / mg tissue)*
Saline	saline	0.33 ± 0.04	0.20 ± 0.04
Verapamil (50 mg/kg)	saline	0.33 ± 0.05 (100%)	0.23 ± 0.04 (115%)
Nifedipine (30 mg/kg)	saline	0.32 ± 0.04 (97%)	0.20 ± 0.02 (100%)
Saline	MDMA	0.18 ± 0.03 (54%) *	0.11 ± 0.03 (55%) *
Verapamil (20 mg/kg)	MDMA	0.18 ± 0.02 (54%)	0.09 ± 0.04 (45%) *
Verapamil (50 mg/kg)	MDMA	0.19 ± 0.04 (57%) *	0.10 ± 0.04 (50%) *
Nifedipine (25 mg/kg)	MDMA	0.13 ± 0.06 (39%) *	0.07 ± 0.03 (35%) *
Nifedipine (30 mg/kg)	MDMA	0.12 ± 0.05 (36%) *	0.08 ± 0.03 (40%) *

* $P < 0.001$, compared to saline control.

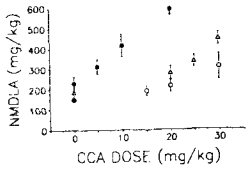

Fig. 1. Dose–response curves for the anticonvulsant activity of CCA's against NMDLA. Separate groups of rats were pretreated i.p. with either saline or one of the CCA's 30 min prior to a constant infusion (via tail vein) of racemic NMDA (NMDLA). Each point represents the mean convulsive threshold for a group of ten animals; the vertical bars represent the S.D. The control convulsive threshold values (in mg/kg NMDLA)) were 186.2 ± 38.2, 166.6 ± 12.7 and 230.4 ± 31.4 for nifedipine, flunarizine and verapamil groups, respectively.

minimal-convulsive threshold in rats (Fig. 1). The anticonvulsant effects of the CCA's were dose-related within the range investigated; verapamil appeared most potent, compared with nifedipine and flunarizine. The ability of the CCA's to antagonize NMDA-induced convulsions suggest that calcium influx is involved and that the translocation of calcium is gated, at least in part, by voltage-sensitive L-type channels.

The ability of the various CCA's to alter the long-term serotonin-depleting effects of MDMA in rat brain are shown in Tables I and II. MDMA by itself produced a significant reduction in the concentration of serotonin in the hippocampus and frontal cortex (40–55 percent of control) 1 week after its administration. Long-term depletions of the serotonergic metabolite, 5-HIAA, paralleled those of the parent amine (data not shown). Pretreatment with flunarizine, however, blocked the serotonin-depleting effects of MDMA (Table I). Haloperidol, a dopamine-receptor blocking drug, was also found to protect against the amine-depleting effects of MDMA (Table I).

By contrast, pretreatment with verapamil or nifedipine, at doses that elevate the NMDLA minimal convulsive threshold by 100–175%, offered no significant protection against the amine-depleting effects of MDMA in either brain region (Table II). Even very high, near lethal doses of verapamil (50 mg/kg) failed to block MDMA-induced neurotoxicity.

TABLE III

Effects of flunarizine or verapamil on methamphetamine-induced stereotypy

Rats were treated with either flunarizine or verapamil 30 min before the administration of a single dose of methamphetamine (6 mg/kg, i.p.) and assessed individually for the presence or absence of stereotypy 30 min later. Flunarizine produced a statistically significant, dose-related suppression of methamphetamine-induced stereotypy, as determined by the method of Litchfield and Wilcoxon[14]. The estimated ED_{50} was 7.6 mg/kg (95% confidence limits: 4–14 mg/kg).

Pretreatment	*Treatment*	*Number of demonstrating stereotypy*	*Stereotypy (%)*
Saline	methamphetamine	6 of 8	75
Flunarizine (5 mg/kg)	methamphetamine	5 of 8	63
Flunarizine (10 mg/kg)	methamphetamine	4 of 8	50
Flunarizine (20 mg/kg)	methamphetamine	2 of 8	25
Saline	methamphetamine	8 of 10	80
Verapamil (20 mg/kg)	methamphetamine	9 of 10	90

In other experiments, we observed that pretreatment with single doses of flunarizine (range: 5–20 mg/kg) reduced the percentage of methamphetamine-treated rats showing stereotypy in a dose-related fashion (Table III). Flunarizine suppressed stereotypy at doses comparable to those found to prevent the toxic effects of MDMA. Verapamil (20 mg/kg) had no significant effect on methamphetamine-induced stereotypy.

The present investigations show that three CCA's, each drawn from a different class of L-type CCA's[26], antagonize the ability of NMDA to produce convulsions in rats. These findings indicate that the CCA's gain access to the CNS compartment after their systemic administration, and provide additional evidence for the proposal that calcium influx via L-type channels plays an important role in the capacity of NMDA to induce convulsions[11]. By contrast, flunarizine was the sole CCA that blocked the long-term, serotonin-depleting effects of MDMA. Moreover, the dose–effect relationships observed for the ability of flunarizine to antagonize either the convulsive actions of NMDA or the toxic effects of MDMA appeared discrepant, because flunarizine provided complete protection at doses that only minimally raised the convulsive threshold. Taken together, the results suggest that an influx of calcium through L-type channels is unlikely to participate in the mechanism by which MDMA damages serotonergic neurons. A similar conclusion was reached in a recent study in which it was reported that flunarizine, but not nimodipine or diltiazam, blocked MDMA-induced reductions in tryptophan hydroxylase activity[10].

The failure of verapamil and nifedipine to block MDMA-induced neurotoxicity in the present studies suggests that the protective effects of flunarizine depend upon an action at CNS sites other than L-type channels. One attractive possibility in this regard is the ability of flunarizine to interact with dopaminergic systems. Previous investigations have established a role for dopamine in the neurotoxic mechanism of action of MDMA[24]. Other studies have shown that flunarizine binds with moderate affinity to striatal D2 receptors and induces alterations in dopaminergic release and metabolism in a manner similar to D2 antagonists, such as sulpiride[5,8,16]. Clinical reports indicate that flunarizine is capable of inducing extrapyramidal symptoms in humans[24]. These data, suggesting that flunarizine is capable of blocking dopamine receptors, gain support from the results of our behavioral studies. We observed that flunarizine, at doses comparable to those found to prevent the toxic effects of MDMA, inhibited the stereotypic effects of methamphetamine, a behavioral paradigm widely believed to reflect an activation of dopaminergic receptors[13]. The findings may provide an explanation for the neuroprotective actions of flunarizine, as pretreatment with the D2 antagonist haloperidol also blocked the serotonin-depleting effects of MDMA in the present studies, confirming the results of an earlier investigation[19]. The issue can not be considered as completely settled, however, because haloperidol has also been reported not to protect against MDMA-induced neurotoxicity[10]. In this regard it is important to note that the pharmacology of flunarizine is complex and includes a variety of effects on voltage-sensitive sodium channels, receptor-gated calcium channels, the dopamine transporter and intracellular calcium dependant enzymes[9,17,25]. The present investigations do not exclude the possibility that one or more of these pharmacological actions may contribute to the neuroprotective effects of flunarizine.

It is generally accepted that NMDA antagonists prevent glutamate-related neuronal damage by blocking the NMDA receptor-linked calcium channel, and to the extent that receptor blockade prevents membrane depolarization, the voltage-sensitive calcium channel as well. NMDA antagonists also prevent amphetamine-induced neurotoxicity, giving rise to the proposal that the toxic effects of these agents, like glutamate, result from calcium influx triggered by NMDA receptor activation. However, because both routes of calcium entry are required for the expression of the convulsive[11], and under certain circumstances the neurotoxic, effects of NMDA[18,20,27], the failure of L-type CCA's to block the toxic effects of MDMA or methamphetamine (Finnegan et al., unpublished results) raise the possibility that the role of the NMDA receptor in amphetamine-induced neurotoxicity may be different from that in glutamate-related neuronal damage. In this regard other mechanisms, such as the reported ability of NMDA antagonists to reduce methamphetamine-induced dopamine release[28], may prove to be more important. Additional studies will be required to clarify the function of the NMDA receptor in the neurotoxic effects of the amphetamines.

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Antinociceptive activity of liposome-entrapped calcitonin by systemic administration in mice

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Porcine calcitonin (pCT) encapsulated in sulfatide-containing reverse-phase evaporation vesicles (REVs) was shown to induce long-lasting and dose-dependent antinociceptive effect by systemic injection in mice. Its antinociceptive activity is found to be dependent on the route of administration. In contrast, neither unencapsulated pCT nor pCT encapsulated in sulfatide-free REVs was effective. These results suggest that pCT entrapped in sulfatide-containing liposomes might be able to cross the blood–brain barrier (BBB) and to produce central antinociception in mice.

Since Pecile et al.[22] discovered the potent analgesia of salmon calcitonin (CT) following intraventricular injection in rabbits, quite a number of studies have been carried out to investigate this effect of CT in animals[2,4,18,27] and humans[7,16]. Being a water-soluble polypeptide, CT is known to be unable to cross the blood–brain barrier (BBB) to enter brain[2,17,18] by itself. Thus only limited methods, such as epidural[7], intracerebroventricular (i.c.v.)[4,18,22,24,27] and subarachnoid[7,16] injections, are available for CT administration. The disadvantages of these routes of administration such as restricted or delayed distribution of injected compounds in the central nervous system (CNS), high level of infectious and deleterious incidence and toxic reactions have been reported[3].

Liposome is one of the promising candidates capable of delivering bioactive materials into desired animal organs. It was reported that several polypeptides and enzymes like horseradish peroxidase were efficiently incorporated into the brain of laboratory animals when entrapped in liposomes composed of phosphatidylcholine (PC), cholesterol (chol) and sulfatide (galactocerebroside 3′-sulfate), apparently by crossing the BBB[19,21,29]. It is therefore interesting for us to know whether bioactive polypeptides such as calcitonin can be encapsulated in liposomes and transported across the BBB to act on the CNS. In this communication, we report the results of a study on the central antinociceptive activity of porcine CT (pCT) entrapped in sulfatide-containing reverse-phase evaporation vesicles (REVs) in mice.

Sulfatide was isolated and purified from human brain according to the methods of Agriga et al.[1] and Ledeen et al.[14]. Briefly, a silicic acid column[26] (Unisil, 200–325 mesh, Clarkson Chemical Co., Williamsport, PA) was utilised to elute the glycolipids with acetone/methanol (9:1, v/v) from the lipid extraction of the Folch lower phase[8] of the brain tissue. The mixture of glycolipids was then applied to a DEAE Sephadex A-25 column (Sigma, USA) and the cerebrosides were removed by chloroform/methanol/water (30:60:8, v/v/v) elution. Sulfatide was obtained as its sodium salt by eluting with methanol/sodium acetate (0.1 M). The purity of the sulfatide obtained was determined by thin-layer chromatography developed with chloroform/methanol/water (65:25:4, v/v/v). The purified sulfatide was found to be greater than 98% pure by the spectrophotometric method of Kean[13].

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