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Research report

3,4-Methylenedioxymethamphetamine ('Ecstasy') promotes the translocation of protein kinase C (PKC): requirement of viable serotonin nerve terminals

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Abstract

The metabolic effects of the neurotoxic, ring-substituted amphetamine 3,4-methylenedioxy-methamphetamine (MDMA or 'Ecstasy') were examined in vivo. In this study, we focused on the ability of MDMA to induce a translocation of the calcium and phospholipid-dependent protein kinase C (PKC) from the cytosol to the cortical plasma membrane. Two injections of MDMA (20 mg/kg; 10 h apart; s.c.) increased the density of membrane bound PKC sites by 48.0% over saline treated animals without mediating a significant change in ligand ([^{3}H]phorbol 12,13 dibutyrate; [^{3}H]PDBu) affinity. Longer drug treatments (8 × 20 mg/kg) induced a lasting (up to 5 days post-treatment) increase in the density of membrane-bound PKC. Prior destruction of cortical 5-HT nerve terminals with *p*-chloroamphetamine (PCA) prevents this effect and suggests that viable 5-HT uptake sites are essential for MDMA-induced PKC translocation. These results demonstrate that MDMA-induced PKC translocation is mediated by viable cortical 5-HT nerve terminals, and that prolonged kinase activation may contribute to MDMA-induced serotonergic neurotoxicity.

Keywords: Serotonin; Serotonin$_2$ receptor; Calcium; Second messenger; Release; Degeneration

1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA or 'Ecstasy') is an amphetamine derivative whose acute psychoactive and long-term neurotoxic effects are dependent on its ability to promote the release of serotonin (5-HT) from presynaptic nerve terminals [6,32,40]. MDMA's other acute biochemical effects include an inhibition of [^{3}H]5-HT uptake and a selective inhibition of monoamine oxidase-A (MAO-A) [25,45,49]. These effects can combine to increase the extracellular concentration of 5-HT in the synapse [22]. Chronic MDMA exposure is damaging to the serotonergic innervation of the cortex as indicated by long-lasting reductions in the density of forebrain 5-HT nerve terminals and uptake sites, as well as histological alterations in the distribution of 5-HT-immunoreactive (5-HT-IR) axons [3,10,19,37]. Although the acute biochemical and neurotoxic effects of MDMA are well known, the metabolic events that result in fiber loss remain unclear.

There is increasing acceptance of the neurotoxic potential associated with disturbances in cellular Ca^{2+} homeostasis. This disregulation has been strongly implicated in the neuropathology produced by ischemia and the excitatory amino acid glutamate [8,46]. Studies performed by Manev et al. [28,29] have shown that these ionic perturbations are related to prolonged ($\approx$ 2 h) translocation and activation of the phospholipid and calcium-dependent protein kinase C (PKC; ATP: proteinphosphotransferase, EC 2.7.1.37) [33,44]. PKC exists inactively as a soluble cytosolic enzyme, but becomes translocated to the plasma membrane in its activated form, bringing it close to many of its substrate proteins [14,48]. PKC translocation is normally a transient event, but can become temporally elongated by extended glutamate receptor stimulation and excessive calcium entry into cultured cerebellar neurons [28,29,34].

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We have shown that both 5-HT and MDMA increase $^{45}Ca^{2+}$ uptake into rat brain synaptosomes [36]. Increases in intracelular Ca^{2+} concentration may occur via influx through voltage or ligand-gated calcium channels, or through its release from intracellular stores and may play a pivitol role in the production of MDMA-induced cell death. MDMA-induced neuropathy in culture and in vivo can be reversed by 5-HT_2 receptor and calcium channel antagonists (ketanserin, MK-801 and nimodipine), suggesting that the toxic event is dependent on disturbances in internal calcium homeostasis mediated via this receptor [2,15,41].

MDMA-mediated increases in intracellular [Ca^{2+}] may be related to an interaction with the central 5-HT_2 receptor; for which both MDMA and 5-HT have similar affinities [3]. Activation of the 5-HT_2 receptor increases intracellular calcium concentration via the G-protein-linked hydrolysis of phosphotidylinositol 4,5-bisphosphate (PIP_2) and the liberation of inositol 1,4,5-trisphosphate (IP_3) [11,12,24]. This receptor has also been shown to mediate the translocation and activation of PKC in several neuronal preparations [47].

The effects of acute and chronic MDMA exposure on the distribution of PKC within the cerebral cortex were investigated in treated and untreated rats. The density of membrane-bound PKC sites were measured via high-affinity [^{3}H]phorbol ester binding to cortical membranes. An increase in membrane-bound PKC is indicative of kinase translocation from the cytosol to the plasma membrane [28,46,48]. Tumor promoting phorbol esters bind PKC with nanomolar affinity, and provide a good representation of the distribution of PKC throughout the brain [50]. The time-course of MDMA-stimulated PKC translocation was compared to the onset of 5-HT terminal degeneration. These results may elucidate some of the metabolic determinants of MDMA-induced neuropathology.

2. Materials and methods

2.1. Animal care and handling

Male Sprague–Dawley rats (weighing between 150 and 200 g; Taconic Farms, PA) were housed one per cage and used for all experiments unless otherwise specified. All animals were maintained on a 12 h light/dark cycle and had free access to food and water. All animal protocols were reviewed and approved by the New York University animal welfare committee.

2.2. In vivo drug treatment

Sixteen (16) rats ($n = 4$ per group) received two subcutaneous injections of either 20 mg/kg of (+)-MDMA (spaced 10 h apart) or vehicle (0.9% saline) for periods ranging from 1 to 4 days. Eight (8) rats received two injections of *p*-chloroamphetamine (PCA; 10 mg/kg × 1/day; s.c) or saline 21 days prior to the beginning of MDMA treatment. 1, 2, 3, 5, or 7 days following the last MDMA injection, the animals were placed under deep anesthesia (CO_2), decapitated, and their brains rapidly removed followed by careful dissection of the cerebral cortex. These brain regions were placed on dry ice and stored at −70° C until use.

2.3. [^{3}H]phorbol 12,13 dibutyrate ([^{3}H]PDBu) binding to treated and untreated tissue

Frozen samples were thawed to +4° C and homogenized in a hypotonic Tris buffer (1:10 by dilution of a normal Tris buffer; (Tris HCl: 38.5 mM, Tris base: 11.5 mM, NaCl: 120 mM, and KCl: 5.6 mM at pH 7.4) using a glass/teflon homogenizer. The tissue was centrifuged at 48,000 × *g* for 20 min and the resultant pellet was resuspended in 40 × v/w in buffer. Forty μl (0.1 mg of protein as assessed by the method of Lowry et al. [27]) of membranes were plated into 96-well plates (Nunc, Denmark) and allowed to equilibrate with 20 μl of either Tris buffer or phorbol 12-myristate 13-acetate (PMA, 3 μM) to determine non-specific binding. Scatchard analyses were performed to determine total binding and the initial [^{3}H]PDBu binding parameters (K_D and B_{max}) using [^{3}H]PDBu (1–40 nM) (New England Nuclear, specific activity 18.6 Ci/mmol) in a total well volume of 200 μl for 45 min at room temperature. One-point determinations (7 nM [^{3}H]PDBu; the approximate K_D obtained from previous experiments)) were used to assay the density of membrane-bound [^{3}H]PDBu binding sites in some experiments using the above protocol. The tissue was harvested onto Titertek filtermats (coated with polyethylimine) using a Titertek cell harvester, and the filters were placed in scintillation vials containing 3.0 ml Liquiscint (National Diagnostics). Samples were counted for 5 min. in a Beckman liquid scintillation counter (efficiency: 44%). CPM data was converted to pmol of [^{3}H]PDBu bound/mg protein.

2.4. Specific [^{3}H]paroxetine binding to cortical membranes

Membrane fractions were prepared as indicated above and were assayed for the density of cortical 5-HT uptake sites. 160 μl of membrane homogenate (0.12 mg of protein) were incubated in the presence of either 40 μl of Tris-HCl buffer (total binding) or 1μM fluoxetine (non-specific binding) for 5 min. 200 μl of [^{3}H]paroxetine (final concentration 0.2 nM; specific activity 15.3 Ci/mmol, New England Nuclear) were added and allowed to equilibrate in the dark for 120

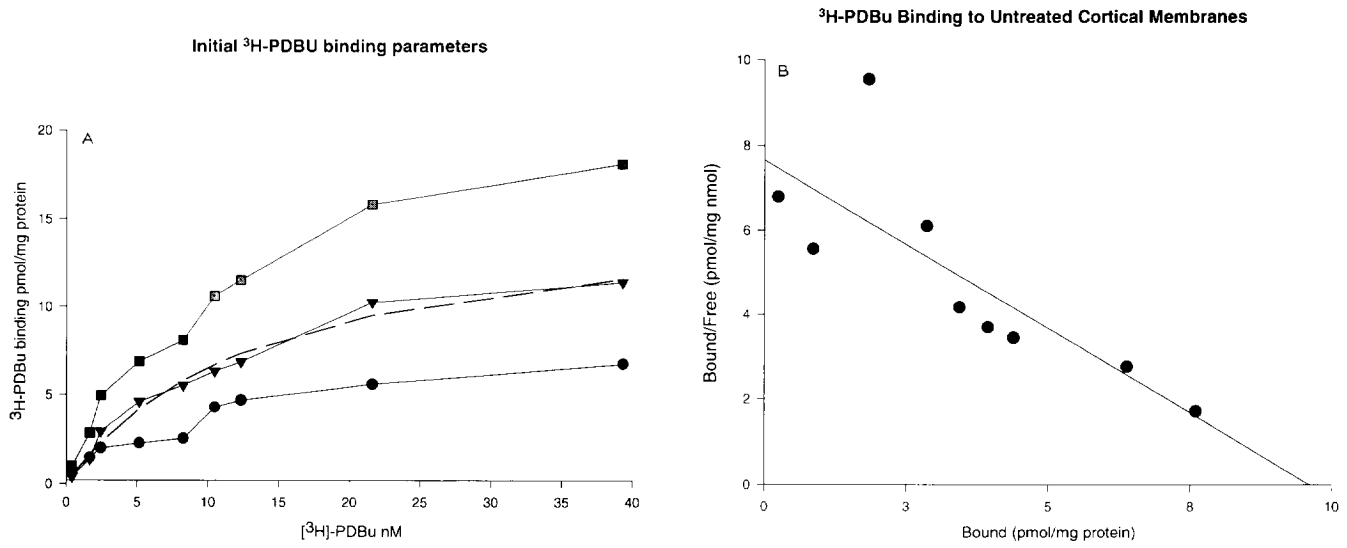

Fig. 1. a: saturation curve of [^{3}H]PDBu binding to untreated rat brain cortical membranes. [^{3}H]PDBu (1–40 nM) was incubated either in the presence of Locke's buffer (total binding: ■) or 3 μM PMA (non-specific: ●) for 45 min. at room temperature. Specific binding (▼) was calculated as the difference between the two curves. The dotted line (—-) represents the best fit line as determine by an iterative curve-fitting program (SigmaPlot for Windows; Jandel Scientific, San Rafael, CA). Each data point is the average ± S.D. of four determinations performed over three experiments ($n = 3$). Results are represented as pmol of [^{3}H]PDBu bound/mg protein. All S.D.'s were less than 8.0% of the calculated mean for all data points. b: Scatchard isotherm transformation of the data contained within the linear binding curve presented in (a). [^{3}H]PDBu (1–40 nM) was incubated either in the presence of Locke's buffer (total binding) or 3 μM PMA (non-specific) for 45 min at room temperature. The data points represent specific binding of [^{3}H]PDBu to untreated rat brain cortical membranes. Each data point is the average ± S.D. of four determinations performed over three experiments ($n = 3$). The data was best-fit by by a one-site model (Hill coefficient: 0.998). Results are represented as the K_D (9.54 ± 0.75 nM) and B_{max} (9.72 ± 1.84 pmol of [^{3}H]PDBu bound/mg protein) of radioligand binding. All S.D.'s were less than 8.0% of the calculated mean for all data points.

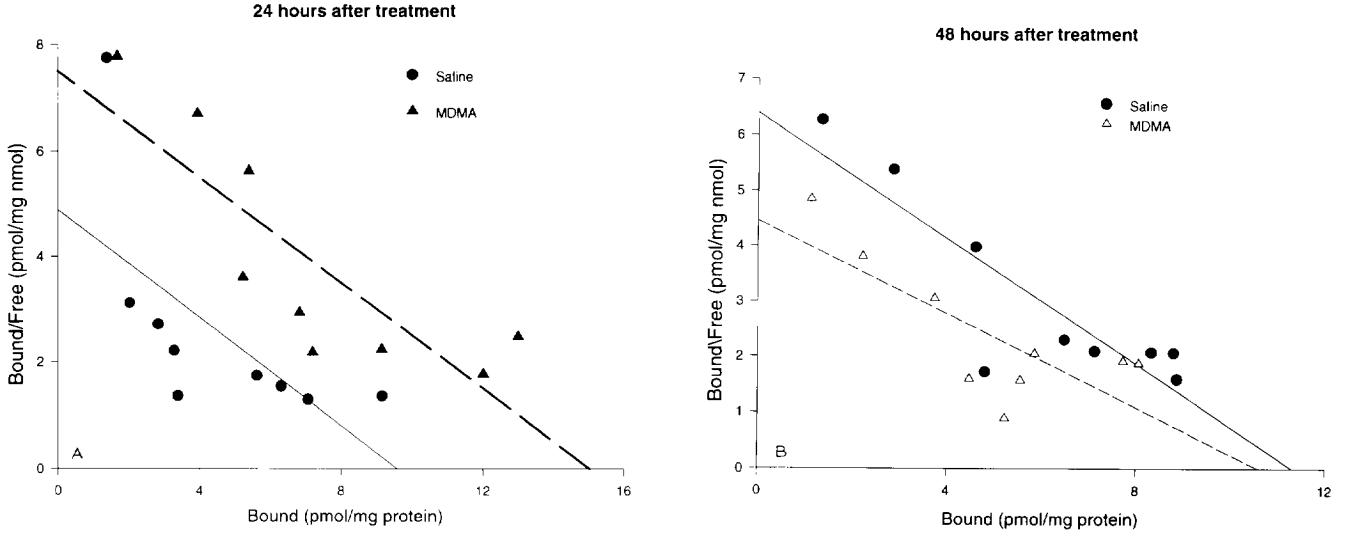

Fig. 2. a: Scatchard isotherm equation describing the specific binding of [^{3}H]PDBu (1–40 nM) to either saline or MDMA treated rat brain corticies. Eight rats received either saline or MDMA (2 × 20 mg/kg; s.c.) 10 h apart before being sacrificed 24 h later ($n = 4$ per group). The binding assay was performed as indicated in the methods section.. 3 μM PMA was used to determine non-specific binding. Each data point is the average ± S.D. from four animals. Saline treated animals showed a $K_D = 9.80 \pm 0.05$ nM and a $B_{max} = 9.53 \pm 0.98$ pmol/mg protein. MDMA-treated animals showed a $K_D = 9.05 \pm 0.19$ nM and a $B_{max} = 14.10 \pm 1.23$ pmol/mg protein. All S.D.'s were less than 8.0% of the calculated mean for all data points. * $P \leq 0.05$ by Student's t-test. b: Scatchard isotherm equation describing the specific binding of [^{3}H]PDBu (1–40 nM) to either saline or MDMA treated rat brain corticies. Eight rats received either saline or MDMA (2 × 20 mg/kg; s.c.) 10 h apart before being sacrificed 48 h later ($n = 4$ per group). The binding assay was performed as indicated in the methods section. 3 μM PMA was used to determine non-specific binding. Saline-treated animals showed a $K_D = 11.80 \pm 1.05$ nM and a $B_{max} = 11.01 \pm 2.01$ pmol/mg protein. MDMA treated animals showed a $K_D = 13.80 \pm 1.26$ nM and a $B_{max} = 10.50 \pm 0.90$ pmol/mg protein. Each data point is the average ± S.D. from four animals. All S.D.'s were less than 8.0% of the calculated mean for all data points.

min at 22° C. The harvesting of membranes and the counting of the bound radioactivity was similar to the method indicated above. CPM data was converted to fmol of [^{3}H]paroxetine bound/mg protein.

2.5. Compounds and chemicals

All chemicals were of reagent grade and were purchased from the following sources:(+)-3,4-methylenedioxymethamphetamine (National Institute on Drug Abuse, Bethesda, MD); fluoxetine HCl (Lilly, Indianapolis, IN); phorbol 12 myristate-13 acetate (PMA), and phorbol 12, 13 dibutyrate (PDBu) (Sigma Co., St. Louis, MO).

2.6. Statistics

[^{3}H]Phorbol ester and [^{3}H]paroxetine binding data were analyzed using an iterative curve-fitting program (LIGAND; [31] and EBDA; [30]). All statistical significance was evaluated using the one-way ANOVA and post-hoc Tukey analysis at a significance level of $P \leq 0.05$. Student's t-test was also used when applicable at $P \leq 0.05$. Results are expressed as mean ± S.D. of four determinations performed over at least three experiments.

3. Results

3.1. Initial parameters of [^{3}H]PDBu binding

Fig. 1a shows that [^{3}H]PDBu binding to cortical membranes is saturable, and can be displaced with the phorbol ester; 12-myristate 13 acetate (PMA) which was used to quantify non-specific binding (Fig. 1a). The initial binding parameters, as determined by the Scatchard isotherm equation, show that this ligand binds to a single population of sites (Hill coefficient: 0.998) with a K_D of 9.54 ± 0.75 nM and a B_{max} of 9.72 ± 1.84 pmol/mg protein (Fig. 1b).

3.2. In vivo translocation of [^{3}H]PDBu binding sites by MDMA

Injecting rats with two doses of MDMA (20 mg/kg, s.c.; 10 h apart) produced a significant increase in the density of membrane-bound PKC binding sites. Twenty-four h after the final injection, the B_{max} of [^{3}H]PDBu binding in MDMA treated animals was 14.10 ± 1.23 pmol/mg protein compared with 9.53 ± 0.98 pmol/mg protein ($n = 4$) for saline (+48.0% over saline) (Fig. 2a). There was no significant change in ligand affinity after drug treatment (saline $K_D = 9.80$ ± 0.05 nM versus MDMA $K_D = 9.05 \pm 0.19$ nM).

No significant difference in [^{3}H]PDBu binding parameters was observed when membrane fractions were assayed 48 h after two MDMA injections (saline: B_{max} = 11.01 ± 2.01 pmol/mg protein vs. MDMA: B_{max} = 10.50 ± 0.90 pmol/mg protein; saline: $K_D = 11.80 \pm$ 1.05 nM versus MDMA: $K_D = 13.80 \pm 1.26$ nM) (Fig. 2b).

Exposure to eight doses of MDMA (over 4 days) also increased membrane bound PKC binding sites (Table 1). This change was observed up to 5 days after the last injection, and was accompanied by no appreciable change in ligand affinity (Table 1). By day 7, the density of membrane bound PKC sites was comparable to saline treated controls (MDMA: $B_{max} = 12.61 \pm 1.51$ vs. saline: $B_{max} = 10.93 \pm 1.33$ pmol/mg protein) (Table 1).

3.3. Effects of serotonergic lesion on [^{3}H]PDBu and [^{3}H]paroxetine binding after MDMA

In order to relate the MDMA-induced increase in cortical PKC binding sites to the presence of 5-HT nerve terminals, animals were pretreated with saline or p-chloroamphetamine (PCA) (2 × 10 mg/kg, s.c) to selectively destroy cortical 5-HT axons (experimental (EXP.) days 1 and 2). MDMA (8 × 20 mg/kg, s.c.) or saline injections were made twice daily (10 h apart), 21 days after the last PCA treatment (EXP. days 23-26).

Table 1
Time course of [^{3}H]PDBu binding to cortical membranes after in vivo MDMA treatment

Treatment ($n = 4$)	Dose (mg/kg) ×2/day	Day of assay after final injection	K_D (nM) Mean ± S.D.	B_{max} (pmol/mg) Mean ± S.D.	B_{max} % increase over control
Saline	0.00	3, 5, 7	9.09 ± 1.27	10.93 ± 1.33	0.00
4-day MDMA	20.0	3	9.60 ± 1.23	22.32 ± 1.61	104.52 [a]
4-day MDMA	20.0	5	7.55 ± 2.00	17.60 ± 2.67	61.43 [a]
4-day MDMA	20.0	7	9.45 ± 0.89	12.61 ± 1.51	15.41

Rat brains were analyzed 3, 5, or 7 days after the final drug exposure. [^{3}H]PDBu (1–40 nM) was incubated either in the presence of Locke's buffer (total binding) or 3 μM PMA (non-specific) for 45 min at room temperature. Results are average ±S.D. from four animals per experimental group. Results are represented as the K_D (nM) and B_{max} (pmol of [^{3}H]PDBu bound/mg protein) of radioligand binding. All S.D.s were less than 8.0% of the calculated mean for all data points.

[a] $P \leq 0.05$ compared to saline controls by Student's t-test.

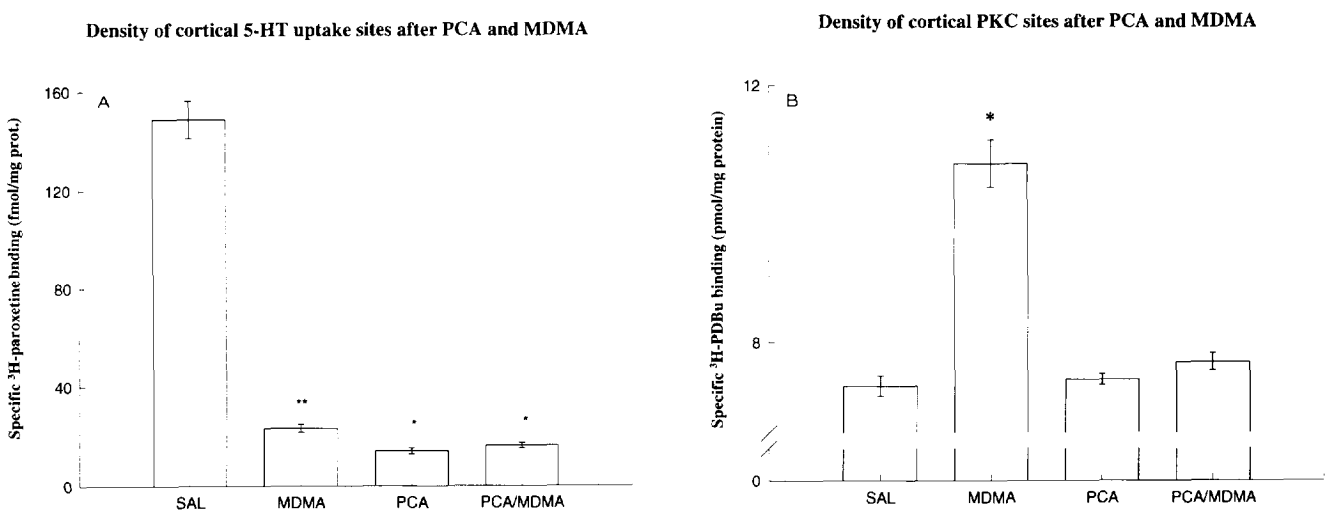

Fig. 3. a: effects of saline, *p*-chloroamphetamine (PCA: 2 × 10 mg/kg s.c), or MDMA (8 × 20 mg/kg s.c) on the specific binding of [^{3}H]paroxetine (0.2 nM) to cerebral cortical membranes on experimental day 29 (see Results section). 1 μM fluoxetine was used to determine non-specific binding Animals were pretreated with either saline or PCA 21 days before the commencement of MDMA injections. Rats received MDMA over four days (2 injections per day) before being sacrificed 3 days after the final treatment. Each bar represents the mean ± S.D. from four animals and each experiment was repeated three times. One-way ANOVA showed significant variance between all four groups (P value = 6.26×10^{-11}; $F = 235.85$, $F_{crit} = 3.49$). * $P \leq 0.001$ when compared to saline controls alone by Tukey post-hoc analysis. ** $P \leq 0.05$ when compared to PCA or PCA/MDMA by Tukey post-hoc analysis. b: effects of saline, *p*-chloroamphetamine (PCA: 2 × 10 mg/kg s.c), or MDMA (8 × 20 mg/kg s.c) on the specific binding of [^{3}H]PDBu (7.0 nM) to cerebral cortical membranes on experimental day 29 (EXP 29) (see results section). 3 μM PMA was used to determine non-specific binding. Animals were pretreated with either saline or PCA 21 days before the commencement of MDMA injections. Rats received MDMA over four days (2 injections per day) before being sacrificed 3 days after the final treatment. Each bar represents the mean ± S.D. from four animals and each experiment was repeated three times. One-way ANOVA showed significant variance between all four groups (P value = 2.23×10^{-4}; $F = 37.25$, $F_{crit} = 3.49$). * $P \leq 0.01$ when compared to all other groups by Tukey post-hoc analysis.

Cortical [^{3}H]paroxetine and [^{3}H]PDBu binding sites were measured 3 days after the last treatment (EXP. day 29) in order to compare the activation of PKC to the density of cortical 5-HT nerve terminals.

Both PCA and MDMA decreased the density of cortical [^{3}H]paroxetine binding sites. PCA reduced the number of terminal 5-HT uptake sites by 94.5% compared to saline treated controls (13.95 ± 1.17 fmol/mg protein vs. 148.80 ± 7.48 fmol/mg protein, respectively, $p \leq 0.001$). MDMA produced a less severe decrease in 5-HT nerve terminal density than PCA (23.14 ± 1.52 fmol/mg vs. 13.95 ± 1.17 fmol/mg protein, respectively; $p \leq 0.05$), and did not augment the reduction in [^{3}H]paroxetine binding sites when given to animals who previously received PCA (PCA: 13.95 ± 1.17 fmol/mg protein; PCA/MDMA: 16.12 ± 1.09 fmol/mg;) (Fig. 3a).

Two injections of PCA produced no significant changes in the density of membrane bound phorbol ester binding sites compared with saline-treated controls (Fig. 3b). MDMA exposure increased the density of [^{3}H]PDBu binding sites in the cerebral cortex by 47.3% over control (SAL: 7.32 ± 0.15 pmol/mg protein vs. MDMA: 10.78 ± 0.37 pmol/mg protein, $P \leq 0.01$). The MDMA-induced changes in PKC binding sites were not observed in animals pretreated with PCA (MDMA: 10.78 ± 0.37 pmol/mg protein; PCA/MDMA: 7.69 ± 0.13 pmol/mg, $P \leq 0.01$) (Fig. 3b). Consequently, MDMA-mediated PKC translocation is not observed in rat cortex after 5-HT terminal loss has already occurred.

4. Discussion

The redistribution of [^{3}H]PDBu binding sites to the plasma membrane is believed to be an indicator of receptor-mediated PKC activation [18,46,47]. Our results indicate that MDMA increases the density of membrane-bound phorbol ester binding sites when administered in vivo (Fig. 2a). This effect of MDMA may be related to its ability to release 5-HT from central serotonergic neurons [23,40]. Compounds known to promote the release of acetylcholine (ACh) and dopamine (DA) from rat brain have also been reported to increase PKC activity, as well as the plasma-membrane (particulate) density of PKC, raising the possibility that MDMA might elicit a similar response through the release of 5-HT [18,21].

Acute exposure to MDMA (2 × 20 mg/kg) produced an increase in cortical [^{3}H]PDBu binding sites that was significant 24 h, but not 48 h after the final injection (Fig. 2a and b). The effect observed at 24 h may indicate PKC activation via 5-HT, and is consis-

tent with the pharmacokinetics of MDMA and the time-course of 5-HT release [42,47]. Rats treated by eight doses of MDMA (20 mg/kg; over 4 days) showed an increase in cortical PKC binding sites for up to 5 days after the final injection (Table 1). Seven days after the cessation of treatment there was no significant difference in the density of membrane-bound PKC sites between the two groups. This observation may reflect the depletion of cytosolic 5-HT by MDMA after repeated drug administrations; either through its release from synaptic endings, inhibition of synthesis, or from the commencement of nerve terminal degeneration [10,43].

The importance of viable 5-HT nerve terminals and the subsequent release of 5-HT was investigated with animals who were exposed to to the serotonin-specific neurotoxin, *p*-chloroamphetamine (PCA) before receiving MDMA 21 days later. Rats pretreated with PCA (10 mg/kg × 2) showed a marked decrease in the density of cortical 5-HT uptake sites, and in those animals, MDMA (8 × 20 mg/kg) was unable to induce a redistribution of [^{3}H]PDBu binding sites. In addition, MDMA did not augment the decrease in cortical [^{3}H]paroxetine binding sites produced by PCA and confirms the results of Berger et al. [6] which suggested that both drugs produce their acute biochemical and neurotoxic effects via similar mechanisms. This raises two possibilities: (1) that PCA pretreatment removed the source of the responsive PKC (e.g. the sensitive PKC pool is contained within 5-HT nerve terminals), or (2) that 5-HT terminal degeneration removed the stimulus (5-HT) that elicits a postsynaptic (receptor-mediated) PKC activation.

Protein kinase C activation has been shown to contribute to vesicular serotonin release and suggests that a pool of this enzyme is readily available in the 5-HT nerve terminal [17]. Amphetamine (AMPH), which is both a DA releaser and neurotoxin, induced the translocation and activation of PKC in vivo ($\geq$ 1 mg/kg) [16,18]. This method of PKC translocation was reported to require intact DA stores and was coupled to cytosolic DA release. The effect of AMPH on PKC activity was attenuated by reducing intracellular DA levels with reserpine and the DA synthesis inhibitor, α-methylparatyrosine (AMPT). In our study, MDMA, a substituted amphetamine, produced a significant increase in the density of membrane-bound PKC sites when given in vivo, and required the presence of viable cortical 5-HT nerve terminals (Table 1). PKC activation within the nerve terminal potentiates the basal and K^+-stimulated release of 5-HT, and may result in further depletion of central 5-HT stores [17,26]. These results indicate that MDMA-induced PKC activation may occur within the 5-HT nerve terminal, and that the interaction of MDMA with a functional 5-HT uptake transporter is required for this effect.

Alternatively, MDMA may activate PKC through the release of 5-HT and the stimualtion of postsynaptic 5-HT receptors linked to this second messenger system (5-HT_2) [11,12]. MDMA exposure in rats has been shown to cause a transient down-regulation of cortical 5-HT_2 sites after chronic exposure [39]. This suggests that MDMA, either itself or through the release of 5-HT, can modulate the density and activity of the 5-HT_2 receptor. Blue et al. [7] observed a close spatial relationship between 5-HT_2 receptors and fine cortical 5-HT-IR axons. These fine axons which innervate the neocortex appear to be selectively susceptible to the neurotoxic effects of MDMA [35]. Recent evidence has suggested that some of MDMA's acute and neurotoxic effects arise from an interaction with the central 5-HT_2 receptor [20]. MDMA-induced toxicity of cultured midbrain raphe cells is attenuated by the selective 5-HT_2 receptor antagonist, ketanserin [2]. Similar effects were observed by Schmidt and co-workers [41], who reported that hyperthermia and long-term reductions in cortical, hippocampal, and striatal 5-HT content induced by MDMA are competitively reversed by pre-treatment with the 5-HT_2 antagonist MDL 11,939.

Our results suggest that MDMA may stimulate PKC translocation through the release of 5-HT, coupled with its ability to inhibit serotonin reuptake and its metabolism by MAO-A [6,25,45]. All three of these processes (occurring simultaneously) could greatly increase the concentration of extracellular 5-HT to levels which may lead to the activation of the 5-HT_2 receptor. Our data supports this point because MDMA was unable to alter membrane PKC density in animals pretreated with PCA, which reduces the MDMA-sensitive (cytosolic) pool of 5-HT (Fig. 2a) [38]. The degeneration of cortical serotonergic nerve terminals produced by substituted amphetamines (SA) (PCA and MDMA) can be prevented by co-treatment with fluoxetine, a 5-HT uptake inhibitor which prevents SA-mediated 5-HT release, or by a combination of reserpine and *p*-chlorophenylalanine (PCPA), a serotonin biosynthesis inhibitor, which depletes central serotonergic stores [5,6,40]. This evidence supports the requirement for 5-HT release in the modulation of PKC density by MDMA.

The destabilization of internal Ca^{2+} homeostasis and the extended translocation and activation of protein kinase C has been shown to occur after prolonged glutamate exposure in primary neuronal cultures (50 μM GLU for 15 min.) [13,28,29]. Both of these metabolic events are key amplification steps that result in excitatory amino acid (EAA)-induced neuronal death, and appear to depend on large increases in $[Ca^{2+}]_i$ [8,9,13]. MDMA-mediated toxicity in rat brain and cultured raphe cells can be attenuated by restricting Ca^{2+} influx, supporting the existence of a calcium-dependent component [1,15]. MDMA also produces a

translocation of PKC to the cortical membrane (this report) which may become temporally elongated through increases in intracellular calcium levels [13,36]. The outcome of these events is a delayed destruction of serotonergic nerve terminals.

This study provides evidence that in vivo MDMA exposure produces a prolonged change in the density of membrane-bound PKC sites in cortical plasma membranes, and that viable 5-HT nerve terminals are necessary for this metabolic event. This response may depend on the release of 5-HT from presynaptic nerve terminals and results in protracted PKC activation and eventual nerve terminal degeneration. It is interesting that MDMA and glutamate-induced cytotoxicity move through similar metabolic intermediates, and that both seem to be dependent on the destabilization of internal calcium homeostasis as a final common pathway. The overall activity of PKC is dependent on intracellular calcium concentration, and therefore may be influenced by its alteration. Future studies need to focus on the identity of the cells (neuronal or glial) which contain the MDMA-sensitive PKC, and the Ca^{2+}-dependent substrates that are influenced by these aberrations in normal cellular metabolism.

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References

[1] Azmitia, E.C., Nimodipine attenuates NMDA- and MDMA-induced toxicity of fetal serotonergic neurons: evidence for a generic model of calcium toxicity. In W.H. Gispen and J. Traber (Eds.), *Nimodipine and Central Nervous System Functions: New Vistas*, Springer-Verlaag, Stuttgart, 1989, pp. 141–159.

[2] Azmitia, E.C., Murphy, R.B. and Whitaker-Azmitia, P.M., MDMA (Ecstasy) effects on cultured serotonergic neurons: evidence for Ca^{2+}-dependent toxicity linked to release, *Brain Res.*, 510 (1990) 97–103.

[3] Battaglia, G., Brooks, B.P., Kulsakdinun, C. and DeSouza, E.B., Pharmacological profile of MDMA (3,4-methylenedioxymethamphetamine) at various brain recognition sites, *Eur. J. Pharmacol.*, 149 (1988) 159–163.

[4] Battaglia, G., Yeh, S.Y., O'Hearn, E., Molliver, M.E., Kuhar, M.J. and DeSouza, E.B., 3,4-Methylenedioxymethamphetamine and 3,4-methylenedioxyamphetamine destroy serotonin terminals in the rat brain: quantification of neurodegeneratiom by measurement of [^{3}H]paroxetine-labled serotonin uptake sites, *J. Pharmacol. Exp. Ther.*, 242 (1987) 911–916.

[5] Berger, U.V., Grzanna, R. and Molliver, M.E., Depletion of serotonin using *p*-chlorophenylalanine (PCPA) and reseroine protects against the neurotoxic effects of *p*-chloroamphetamine (PCA) in the brain, *Exp. Neurol.*, 103 (1989) 111–115.

[6] Berger, U.V., Gu, X.F. and Azmitia, E.C., The substituted amphetamines 3,4-methylenedioxymethamphetamine, methamphetamine, *p*-chloroamphetamine and fenfluramine induce 5-hydroxytryptamine release via a common mechanism blocked by fluoxetine and cocaine, *Eur. J. Pharmacol.*, 215 (1992) 153–160.

[7] Blue, M.E., Yagaloff, K.A., Mamounas, L.A., Hartig, P.R. and Molliver, M.E., Corresponsence between 5-HT_2 receptors and serotonergic axon in rat neocortex, *Brain Res.*, 453 (1988) 315–328.

[8] Choi, D.W., Calcium mediated neurotoxicity: relationship to specific channel types and role in ischemic damage, *Trends Neurosci.*, 11 (1988) 465–469.

[9] Choi, D.W. and Rothman, S.M., The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death, *Annu. Rev. Neurosci.*, 13 (1990) 171–182.

[10] Commins, D.L., Vosmer, G., Virus, R.M., Wollverton, W.L., Schuster, C.R. and Seiden, L.S., Biochemical and histological evidence that methylenedioxymethamphetamine (MDMA) is toxic to the rat brain, *J. Pharmacol. Exp. Ther.*, 241 (1987) 338–345.

[11] Conn, P.J. and Sanders-Bush, E., Serotonin-stimulated phosphoinositide turnover: mediation by the S2 binding site in rat cerebral cortex but not in subcortical regions, *J. Pharmacol. Exp. Ther.*, 234 (1985) 195–203.

[12] Conn, P.J. and Sanders-Bush, E., Regulation of serotonin-stimulated phosphoinositide hydrolysis: relation to the serotonin 5-HT_2 binding site, *J. Neurosci.*, 6 (1986) 3669–3675.

[13] DeErasquin, G.A., Manev, H., Guidotti, A., Costa, E. and Brooker, G., Gangliosides normalize distorted single-cell intracellular free Ca^{2+} dynamics after toxic doses of glutamate in cerebellar granule cells, *Proc. Natl. Acad. Sci.*, 87 (1990) 8017–8021.

[14] Dekker, L.V., DeGraan, P.N.E., Oestreicher, A.B., Versteeg, D.H.G. and Gispen, W.H., Inhibition of noradrenaline release by antibodies to B-50 (GAP-43), *Nature*, 342 (1989) 74–76.

[15] Farfel, G.M., Vosmer, G.L. and Seiden, L.S., The *N*-methyl-D-aspartate antagonist MK-801 protects against serotonin depletions induced by methamphetamine, 3,4-methylenedioxymethamphetamine and *p*-chloroamphetamine, *Brain Res.*, 595 (1992) 121–128.

[16] Fisher, J.F. and Cho, A.K., Chemical release of dopamine from striatal homogenates: evidence for an exchange diffusion model, *J. Pharmacol. Exp. Ther.*, 208 (1979) 203–209.

[17] Gandhi, V.C. and Jones, D.J., G-protein modulation of [^{3}H]-serotonin release through the L-type calcium channel, *Ann. NY Acad. Sci.*, 635 (1991) 455–458.

[18] Giambalvo, C.T., Protein kinase C and dopamine transport—1. Effects of amphetamine in vivo, *Neuropharmacology*, 31 (1992) 1201–1210.

[19] Gibb, J.W., Johnson, M., Stone, D. and Hanson, G.R., MDMA: historical perspectives, *Ann. NY Acad. Sci.*, 600 (1990) 601–611.

[20] Glennon, R.A., Teitler, M. and McKenney, J.D., Evidence for 5-HT_2 involvent in the mechanism of action of hallucinogenic agents, *Life. Sci.*, 35 (1984) 2505–2511.

[21] Govoni, S., Battaini, F., Lucchi, L., Pascale, A. and Trabucchi, M., PKC translocation in rat brain cortex is promoted in vivo and in vitro by α-glycerylphosphorylcholine, a cognition enhancing drug, *Ann. NY Acad. Sci.*, 695 (1993) 307–310.

[22] Gu, X.F., *Integrative Release of 5-HT from Cultured Raphe Neurons*, Ph.D. Dissertation, New York University New York, 1993.

[23] Johnson, M.P., Hoffman, A.J. and Nichols, D.E., Effects of the enantiomers of MDA, MDMA, and related analogues on

[^{3}H]serotonin and [^{3}H]dopamine release from superfused rat brain slices, *Eur. J. Pharmacol.*, 132 (1986) 269–276.

[24] Kendall, D.A. and Nahorski, S.R., 5-HT-stimulated inositol phopholipid hydrolysis in rat cerebral cortex slices: pharmacological charecterization and effects of antidepressants, *J. Pharmacol. Exp. Ther.*, 233 (1985) 473–479.

[25] Kokotos Leonardi, E.T. and Azmitia, E.C., MDMA (Ecstasy) inhibition of MAO type A and type B: comparisons with fenfluramine and fluoxetine (Prozac), *Neuropsychopharmacology*, 10 (1994) 231–238.

[26] Kramer, H.K., Chiopelas, E.P., Akbari, H.M. and Azmitia, E.C., L-type Ca^{2+} channel antagonists enhance neurotransmitter release, *Soc. Neurosci. Abstr.*, 18 (1992) 435.

[27] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, 193 (1951) 265–275.

[28] Manev, H., Favaron, M., Alho, H., Bertolino, M., Guidotti, A. and Costa, E., Gangliosides and NMDA-sensitive glutamate receptor antagonists prevent glutamate neurotoxicity via different mechanisms, *Soc. Neurosci. Abstr.*, 14 (1988) 747.

[29] Manev, H., Favaron, M., Guidotti, A. and Costa, E., Delayed increase of Ca^{2+} influx elicited by glutamate: role in neuronal death, *Mol. Pharmacol.*, 36 (1989) 106–112.

[30] McPherson, G.A., A practical computer based approach to the analysis of radioligand binding experiments, *Computer Prog. Biomed.*, 17 (1983) 107–114.

[31] Munson, P.J. and Rodbard, D., LIGAND: a versitile coputerized approach for characterization of ligand binding systems, *Anal. Biochem.*, 107 (1980) 220–239.

[32] Nichols, D.E., Lloyd, D.H., Hoffman, A.J., Nichols, M.B. and Yim, G.K., Effects of certain hallucinogenic amphetamine analogues on the release of [^{3}H]-serotonin from rat brain synaptosomes, *J. Med. Chem.*, 25 (1982) 530–535.

[33] Nishizuka, Y., Studies and perspectives of protein kinase C, *Science*, 233 (1986) 305–312.

[34] Ogura, A., Miyamoto, M. and Kudo, Y., Neuronal death in vitro: parallelism between surviability of hippocampal neurons and sustained elevation of cytosolic Ca^{2+} after exposure to glutamate receptor agonists, *Exp. Brain Res.*, 73 (1988) 447–458.

[35] O'Hearn, E., Battaglia, G., DeSouza, E.B., Kuhar, M.J. and Molliver, M.E., Methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA) cause selective ablation of serotonin axon terminals in forebrain: immunocytochemical evidence for neurotoxicity, *J. Neurosci.*, 8 (1988) 2788–2803.

[36] Park, W.P. and Azmitia, E.C., 5-HT, MDMA (Ecstasy), and nimodipine effects on $^{45}Ca^{2+}$-uptake into rat brain synaptosomes, *Ann. NY Acad. Sci.*, 635 (1991) 438–442.

[37] Ricaurte, G.A., DeLanney, L.E., Weiner, S.G. and Langston, J.W., Toxic effect of MDMA on central serotonergic neurons in the primate: importance of route and frequency of drug administration, *Brain Res.*, 446 (1988) 165–168.

[38] Sanders-Bush, E., Bushing, J.A. and Sulser, F., Long-term effects of *p*-chloroamphetamine and related drugs on central serotonergic mechanisms, *J. Pharmacol. Exp. Ther.*, 192 (1975) 33–41.

[39] Scheffel, U., Lever, J.R., Stathis, M. and Ricaurte, G.A., Repeated administration of MDMA causes transient down-regulation of serotonin 5-HT_2 receptors, *Neuropharmacology*, 31 (1992) 881–894.

[40] Schmidt, C.J., Neurotoxicity of the psychedelic amphetamine, methylenedioxymethamphetamine, *J. Pharmacol. Exp. Ther.*, 240 (1987) 1–7.

[41] Schmidt, C.J., Black, C.K., Abbate, G.M. and Taylor, V.L., Methylenedioxymethamphetamine-induced hyperthermia and neurotoxicity are independently mediated by 5-HT2 receptors, *Brain Res.*, 529 (1990) 85–90.

[42] Schmidt, C.J., Levin, T.A. and Lovenberg, W., In vitro and in vivo neurochemical effects of methylenedioxymethamphetamine on striatal monoaminergic systems in the rat brain, *Biochem. Pharmacol.*, 36 (1987) 747–755.

[43] Schmidt, C.J. and Taylor, V.L., Depression of rat brain tryptophan hydroxylase activity following the acute administraion of methylenedioxymethamphetamine, *Biochem. Pharmacol.*, 36 (1987) 4095–4102.

[44] Sladeczek, F., Pin, J.P. and Recasens, M., Glutamate stimulates inositol phosphate formation in striatal neurones, *Nature*, 317 (1985) 717–719.

[45] Steele, T.D., Nichols, D.E. and Yim, G.K.W., Stereochemical effects of 3,4-methylenedioxymethamphetamine (MDMA) and related amphetamine derivatives on inhibition of uptake of ^{3}H-monoamines into synaptosomes of different regions of rat brain, *Biochem. Pharmacol.*, 36 (1987) 2297–2303.

[46] Vaccarino, F., Guidotti, A. and Costa, E., Ganglioside inhibition of glutamate-mediated protein kinase C translocation in primary cultures of cerebellar neurons, *Proc. Natl. Acad. Sci.*, 84 (1987) 8707–8711.

[47] Wang, H.-Y. and Friedman, E., Central 5-hydroxytryptamine receptor-linked protein kinase C translocation: a functional postsynaptic signal transduction system, *Mol. Pharmacol.*, 37 (1990) 75–79.

[48] Wolf, M., Cuatrecasas, P. and Sahyoun, N., Interaction of protein kinase C with membranes is regulated by Ca^{++}, phorbol ester, and ATP, *J. Biol. Chem.*, 260 (1985) 15718–15722.

[49] Wong, D.T., Horng, J.S. and Fuller, R.W., Kinetics of serotonin accumulation into synaptosomes of rat brain, effects of amphetamine a chloroamphetamines, *Biochem. Pharmacol.*, 22 (1973) 311–320.

[50] Worley, P.F., Baraban, J.M., and Snyder, S.H., Heterogeneous localization of protein kinase C in rat brain: autoradiographic analysis of phorbol ester receptor binding, *J. Neurosci.*, 6 (1986) 199–207.