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Integrative transporter-mediated release from cytoplasmic and vesicular 5-hydroxytryptamine stores in cultured neurons

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The direct effects of 3,4-methylenedioxymethamphetamine (MDMA) and p-chloroamphetamine (PCA) were studied in microculture of fetal 5-hydroxytryptamine (5-HT) neurons. Both MDMA and PCA released 5-HT with the potency of PCA > MDMA by a mechanism inhibited by fluoxetine, an inhibitor of the 5-HT transporter. The transporter-mediated release by MDMA and PCA reduced intracellular stores of 5-HT. Both MDMA and PCA inhibit MAO-A activities, which also contributes to the increase of extracellular 5-HT levels. Deprenyl (10^{-7} M) increased the amount of intracellular 5-HT and potentiated the MDMA- or PCA-induced release of 5-HT. Conversely, reserpine (10^{-9} M) reduced the intracellular 5-HT levels and attenuated the transporter-mediated release. In addition, MDMA- or PCA-mediated release was attenuated by nimodipine (10^{-8} M), an L-type Ca^{2+} channel antagonist. Our results indicate that MDMA- or PCA-induced release of 5-HT occurs from the cytoplasm to the media through the 5-HT transporter, and that the release may incorporate 5-HT from the vesicular stores.

MDMA (3,4-methylenedioxymethamphetamine); Parachloroamphetamine; Fluoxetine; Deprenyl; Clorgyline; Nimodipine; Reserpine

1. Introduction

Substituted amphetamines such as 3,4-methylenedioxymethamphetamine (MDMA) and p-chloroamphetamine (PCA) have acute effects on the release of 5-hydroxytryptamine (5-HT) from presynaptic terminals (Nichols et al., 1982; Fitzgerald and Reid, 1990; Kehne et al., 1992), inhibition of 5-HT uptake by synaptosomes (Hekmatpanah and Peroutka, 1990; Johnson et al; 1991) and reduction of brain monoamine oxidase (MAO-A) activity (Fuller et al., 1975; Kokotos-Leonardi et al., 1992). In chronic studies, these compounds cause neurotoxicity of serotonergic neurons (Schmidt et al., 1987; Ross, 1976). The effects are mainly represented as follows: long-term reduction in brain levels of serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) (Stone et al., 1986; Schmidt et al., 1987; Axt and Seiden, 1990), reduction in the activity of the synthetic enzyme, tryptophan hydroxylase (Stone et al., 1986; Gibb et al., 1990), reduction in the uptake carrier sites (Battaglia et al., 1987; Nash et al., 1991; Dewar et al., 1992) and loss of 5-HT immunoreactive fibers (Scallet et al., 1988; Mamounas and Molliver, 1988; O'Hearn et al., 1988).

However, questions concerning the mechanism of action of the substituted amphetamines and the possible existence of a peripheral factor in contributing to their toxic response in serotonergic neurons have been raised. Both in vivo and in vitro studies have provided evidence that the release of 5-HT may be correlated with neurotoxicity. For example, inhibitors of 5-HT reuptake block the effects of MDMA on both the acute release and long term neurotoxicity (Fuller et al., 1965; Schmidt, 1987; Hekmatpanah and Peroutka, 1990). In addition, pretreatment with drugs which deplete the storage of 5-HT produce an attenuated neurotoxic response to PCA (Berger et al., 1989). Previous tissue culture studies of fetal raphe brainstem are consistent with the in vivo findings. MDMA was found to produce neuropathological changes in fetal serotonergic neurons at a concentration of 10^{-5} M after a single application. This effect was blocked by fluoxetine, a specific 5-HT reuptake blocker, by several drugs which affect presynaptic 5-HT release and by the L-type Ca^{2+} channel antagonist, nimodipine (Azmitia et al., 1990). However, the significance of these findings is compromised by the lack of information concerning the

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direct actions of MDMA on 5-HT release and metabolism in cultured neurons.

We here report a novel system combining tissue culture of fetal raphe brainstem with HPLC-EC for investigating the direct neurotoxicity of serotonergic drugs such as MDMA and PCA. The advantage of this system is that it provides a closed system in which the levels and metabolites of serotonin can be followed without complications from other sources such as serum, glia cells, peripheral organs and endocrine systems. The sequences of the experiments were as follows. First, the ability of MDMA and PCA to release 5-HT from the culture neurons was tested by measuring media 5-HT levels after application of MDMA and PCA at 10^{-5} M for various periods (30 min, 1, 3, 6 and 48 h). Second, the increase of 5-HT levels in the media produced by MDMA or PCA was tested together with (1) a specific serotonergic uptake blocker, fluoxetine (Wong et al., 1974) and (2) the MAO-A inhibitor, clorgyline (Johnston, 1968) and MAO-B inhibitor, deprenyl (Yang and Neff, 1974). MAO-B is located inside serotonergic neurons and MAO-A is found outside serotonergic neurons (Westlund et al., 1985). Finally, the involvement of vesicular store of 5-HT in MDMA- or PCA-induced release of 5-HT was studied by using (1) reserpine, a depleting agent of 5-HT from vesicular stores (Brodie et al., 1964); (2) nimodipine (L-type Ca^{2+}-channel blocker; Towart and Kazda, 1979).

2. Materials and methods

2.1. Brainstem raphe culture

Embryos at 14-days of gestation were quickly removed from timed pregnant Sprague-Dawley rats certified pathogen-free (Taconic Farms, PA) by Cesarean section using antiseptic procedures, and transferred to ice-cold D1-solution containing magnesium; calcium-free minimal essential medium (MEM); and the antibiotics penicillin and streptomycin (Azmitia et al., 1991). A rostral rhombencephalon and caudal mesencephalon tegmental slice was dissected and transferred to 0.5 ml of complete neuronal media (CNM: 1.6% glucose, 1% non-essential amino acids and 7.5% of fetal bovine serum), minced and gently agitated by repeated trituration. The cell suspension was then brought up to 1.5 ml/fetus with CNM and plated at a initial plating density of 0.9×10^6 cells/cm^2 onto 96-well plates (Nunc, Denmark) previously coated with poly-D-lysine (15 μg/ml, 70000 MW, Sigma). The cultures were maintained at temperature of 37°C; air/ CO_2 of 95%/5% and humidity of 98%.

Forty-eight hours later, 5-fluoro-2′-deoxyuridine (FUDR; 2 mg/ml) and uridine (5 mg/ml) were added for a period of 24 h to suppress the proliferation of glia cells and fresh serum-free media (SFM; transferrin 5 μM; insulin 5 mg/ml; putrescine 0.2 mM; corticosterone 0.2 nM; selenite 0.15 nM; MEM; glucose 1% and non-essential amino acid 1%) added. After culturing cells in SFM for 72 h, the media was replaced with 100 μl of freshly-made SFM with the test drugs. The media (60 μl from each well) was then collected at a designed period and stored at −70°C until measurement by HPLC.

To measure the tissue levels of 5-HT, the culture media was removed and 120 μl of 0.1 N perchloric acid was added. The plate was shaken at 7°C for 1 h and the media stored at −70°C.

2.2. HPLC-EC measurements

The mobile phase consisted of 0.15 M chloroacetic acid, 0.12 M NaOH, 0.18 mM EDTA, 6% acetonitrile and 1.0 mM sodium octanesulfate, pH 3.5 (Auerbach et al., 1989). It was delivered by a pump (model 510, Waters. Malford, MA) at a flow rate of 0.9 ml/min onto a 10 × 3 cm chromatography column with ODS 3 μm packing (BAS Inc., W. Lafayette, IN). Samples (20 μl of each) were automatically injected (model 712, Waters. Malford, MA) and analyzed using a dual potentiostat electrochemical detector (model 400 EG & G, Princeton Applied Research Corp. Princeton, NJ) at potential of 610 and 490 mV relative to an Ag/AgCl reference electrode. Identification and quantification of samples were achieved by comparison of retention time and peak area to a standard solution containing a serial of dilutions of 5-HT. The detection limit for 5-HT was approximately 5 pg on a signal-to-noise ratio of 3:1.

Media samples were thawed and mixed with an equal volume of 0.2 N perchloric acid before 20 μl were injected into the analytic column. The signals from the EC detector were captured and integrated by using an 810 baseline program. Media 5-HT levels were expressed as pmol/10 μl while tissue 5-HT levels were expressed as pmol/mg of protein measured by Biorad assay. Analysis of variance was done by using ANOVA followed by a post-hoc comparison (Tukey's test) if not otherwise specified in the text. Every set of experiments was repeated at least two times.

2.3. Drugs

Sources of drugs: all drugs were from Sigma Chemical Company (St. Louis, MO), except MDMA.HCl was from the National Institute of Drug Abuse (Washington, DC), fluoxetine.HCl from Eli Lilly Company (Indianapolis, IN), nimodipine from Miles Research Lab (New Haven, CT) and deprenyl from Research Biochemical Incorp. (Natick, MA).

3. Results

3.1. Time course of MDMA and PCA on inducing release of 5-HT

In control conditions, the amount of 5-HT in serum-free media from the 5-day raphe cultures was undetectable. Solutions containing 7.5% fetal bovine serum were not used since they contained 0.66 pmol/10 μl (6.6×10^{-8} M) levels of 5-HT.

The effects of MDMA (10^{-5} M) and PCA (10^{-5} M) on the media levels of 5-HT were measured at 30 min, 1, 3, 6 and 48 h (fig. 1). Both MDMA and PCA produced significant levels of 5-HT within 1 h (0.23 ± 0.003 and 0.59 ± 0.002 pmol/10 μl respectively). Maximal 5-HT levels in the media were seen after 6 h with MDMA (1.65 ± 0.001 pmol/10 μl) but were still increasing at 48 h after PCA treatment (3.69 ± 0.001 pmol/10 μl; fig. 1).

3.2. Effect of fluoxetine on MDMA- and PCA-induced release of 5-HT

The effect of a specific 5-HT uptake blocker, fluoxetine, was tested in cultures with or without MDMA and PCA. Fluoxetine, at 10^{-7} M had no significant effects on the 5-HT levels in culture. In another series of experiments, fluoxetine (10^{-7} M) or vehicle was added 20 min before MDMA or PCA at 10^{-5} M. After 6 h of exposure, MDMA- and PCA-induced release of 5-HT were significantly blocked by fluoxetine by 50 and 19% respectively (fig. 2).

3.3. Effect of deprenyl and clorgyline on MDMA- and PCA-induced release of 5-HT

Deprenyl (10^{-7} M), an MAO-B inhibitor, had no effect on the media levels of 5-HT after 24 h (fig. 3).

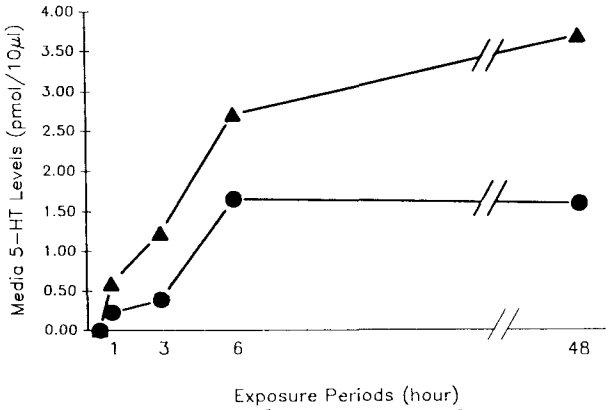

Fig. 1. Effect of MDMA (10^{-5} M) and PCA (10^{-5} M) on releasing of 5-HT into 5-day old, serum-free raphe cell culture media after various periods of exposure to the drugs (30 min, 1, 3, 6 and 48 h). Each data point was expressed as mean ± S.E.M of 5-HT levels (pmol/10 μl; n = 4). MDMA (filled circle); PCA (filled triangle).

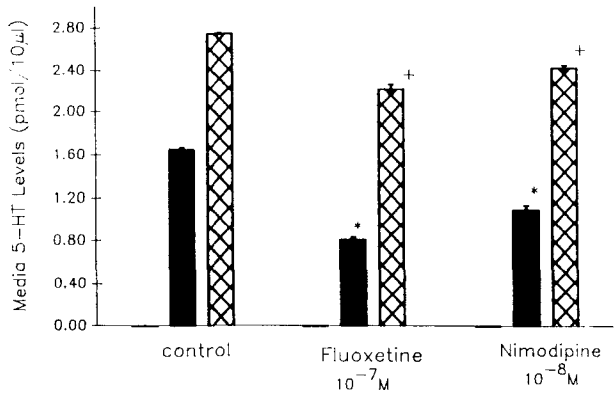

Fig. 2. Inhibitory effects of fluoxetine (10^{-7} M) and nimodipine (10^{-8} M) on MDMA (filled bars)- and PCA (hatched bars)-induced release of 5-HT into the culture media after 6 h of exposure to the drugs. Each bar represented as mean ± S.E.M (media 5-HT levels in pmol/10 μl; n = 4). * $P < 0.01$ as compared to MDMA only treated cultures; $^{+}$ $P < 0.01$ as compared to PCA only treated cultures, $F = 1435.00$, df = 8, ANOVA with Tukey's tests.

Deprenyl (10^{-7} M), added 20 min before MDMA or PCA, increased the amount of 5-HT released into the media by both MDMA and PCA after 24 h of exposure (fig. 3). It augmented MDMA-induced release of media 5-HT levels by 78%, and PCA-induced release of media 5-HT levels by 18%.

Clorgyline (10^{-7} M), an MAO-A inhibitor, produced a large increase in media 5-HT levels (4.1 ± 0.01 pmol/10 μl) by itself. However, the addition of either MDMA or PCA to the cultures containing clorgyline produced no additive increase in 5-HT levels after 24 h compared to clorgyline alone and MDMA or PCA alone cultures (fig. 3).

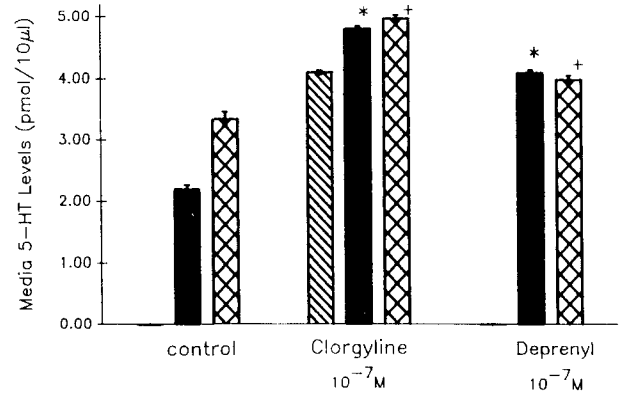

Fig. 3. Effects of clorgyline (10^{-7} M; diagonal bar) and deprenyl (10^{-7} M) on MDMA (filled bars)- and PCA (hatched bars)-induced release of 5-HT into 5-day old serum-free culture media. Media 5-HT levels (pmol/10 μl) were measured 24 h after cultures were exposed to the drugs. Each bar represented as mean ± S.E.M (n = 4). * $P < 0.01$ as compared to MDMA only treated cultures; $^{+}$ $P < 0.01$ as compared to PCA only treated cultures, $F = 1051.40$, df = 8, ANOVA with Tukey's test.

3.4. Effect of reserpine and deprenyl on MDMA- and PCA-induced release of 5-HT

Before testing the effects of reserpine on 5-HT release, the intracellular levels of 5-HT were measured to test the efficiency of reserpine to deplete vesicular stores of 5-HT. The cellular content of 5-HT present in reserpine (10^{-9} M)-treated cultures after 48 h (0.66 ± 0.008 pmol/mg protein) was reduced by 78% as compared to control cultures (3.0 ± 0.12 pmol/mg protein; $P < 0.001$; Student's t-test).

Reserpine (10^{-9} M), added 20 min before MDMA or PCA, attenuated both MDMA- and PCA-induced release of 5-HT (fig. 4). Reserpine decreased MDMA-induced release of 5-HT by 23% and PCA-induced release of 5-HT by 48%.

The effects of reserpine and deprenyl on intracellular 5-HT levels were tested with and without MDMA. As shown in table 1, cultures were pretreated with the above agents 20 min earlier than MDMA (10^{-5} M). Deprenyl (10^{-7} M) after 24 h increased intracellular 5-HT levels in control cultures by 72% and in reserpinized cultures by 140%. The absolute increase in 5-HT levels by deprenyl was greater in control cultures than in reserpine-treated cultures (1.88 vs. 1.16 pmol/mg protein). On the other hand, reserpine after 24 h decreased 5-HT levels in control cultures by 67% and in deprenyl-pretreated cultures by 55%. Again the absolute decrease of 5-HT levels by reserpine was greater in cultures pretreated with deprenyl than in control cultures (2.39 vs. 1.67 pmol/mg protein). The release of 5-HT by MDMA was enhanced by deprenyl while attenuated by reserpine. However, the attenuation produced by reserpine was much less if deprenyl was present in reserpine-treated cultures (table 1, last column). Furthermore, intracellular 5-HT levels were lower in cultures with MDMA and deprenyl than those in cultures with deprenyl only (1.2 vs. 4.38 pmol/mg protein; table 1).

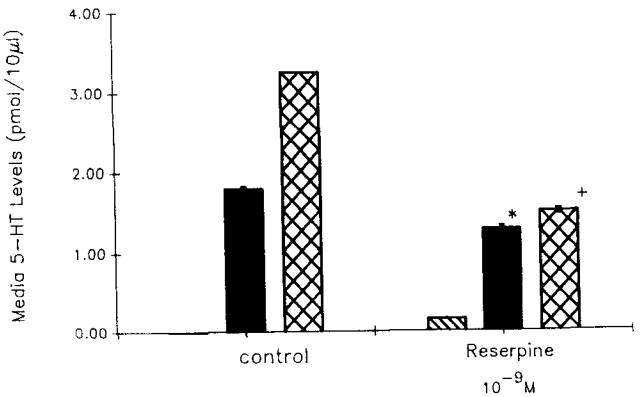

Fig. 4. Effect of reserpine (10^{-9} M) (diagonal bar) on MDMA (filled bar)- and PCA (hatched bar)-induced release of 5-HT into culture media. Media 5-HT levels (pmol/10 μl) were measured 6 h after the exposure of the cultures to the drugs. Each bar represented as mean ± S.E.M (n = 4). * $P < 0.01$ as compared to MDMA only treated cultures; + $P < 0.01$ as compared to PCA only treated cultures, F = 599.22, df = 5, ANOVA with Tukey's tests.

TABLE 1

Effect of different agents (deprenyl 10^{-7} M, reserpine 10^{-9} M and a combination of deprenyl and reserpine) on intracellular 5-HT levels either in control cultures or in MDMA (10^{-5} M)-treated cultures.

Intracellular 5-HT levels (pmol/mg protein) were measured 24 h after the cultures were exposed to the drugs. Data in the fourth column represent the amount of 5-HT released by MDMA. Each data point was represented as mean ± S.E.M (n = 4).

	w/o MDMA (A)	w/ MDMA 10^{-5} M (B)	(A)–(B)
Control	2.500 ± 0.044	1.690 ± 0.052	0.81
Deprenyl 10^{-7} M	4.380 ± 0.020 [a]	1.200 ± 0.028 [b]	3.10
Reserpine 10^{-9} M	0.830 ± 0.024 [a]	0.360 ± 0.020 [b]	0.47
Deprenyl + reserpine	1.990 ± 0.088 [a]	0.020 ± 0.010 [b]	1.97

[a] $P < 0.01$ among the groups in column II, F = 169.32, df = 3; [b] $P < 0.01$ among groups in column III, F = 106.31, df = 3. Variances were analyzed by using ANOVA with Tukey's test.

3.5. Effect of nimodipine on MDMA- and PCA-induced release of 5-HT

To determine whether MDMA- and PCA-induced release of media 5-HT were dependent on a voltage sensitive Ca^{2+} channel, nimodipine was used. After cells were exposed to drugs for 6 h (nimodipine added 20 min earlier), nimodipine (10^{-8} M) was found to significantly attenuate MDMA- and PCA-induced release of 5-HT (24 and 14% respectively; fig. 2).

4. Discussion

4.1. MDMA- and PCA-induced 5-HT release occurs through 5-HT transporter

In our study, MDMA and PCA were found to cause increase of media 5-HT levels in culture after 1 h of exposure. The cultures were maintained in serum-free media and treated with FUDR to kill dividing cells (e.g. astrocytes). This extracellular increase of 5-HT continued progressively over 48 h with PCA (fig. 1), while maximal increase caused by MDMA was stable after 6 h. The continual rise of 5-HT of cells exposed to PCA for 48 h could be due to cell death. In fact, other studies have shown that chronic exposure of

cultured neurons to MDMA, PCA or 5-HT itself may be deleterious (Whitaker-Azmitia and Azmitia, 1986; Azmitia et al., 1990).

Our data supported the idea that the release of intracellular 5-HT by MDMA and PCA occurs via the 5-HT transporter molecule. Fluoxetine (10^{-7} M) pretreatment significantly blocked the amount of extracellular 5-HT released by MDMA and PCA (19–50% of control; fig. 2). This finding is consistent with fluoxetine inhibition of the MDMA-induced decrease in brain 5-HT levels in the intact animal (Schmidt et al., 1987), and of the MDMA- and PCA-induced release of [^{3}H]5-HT from adult synaptosomes (Nichols et al., 1982; Berger et al., 1992). Recent reports have also shown that there is no difference in MDMA-induced release of [3H]5-HT from either the fetal or the adult synaptosomes (Bell et al., 1992). Thus, fetal cultured neurons respond to substituted amphetamine in a similar way as the adult 5-HT neurons.

4.2. Interactions of intracellular and extracellular 5-HT with specific MAO-inhibitors

Monoamine oxidase (MAO) exists in two forms, MAO-A and MAO-B, which have different sensitivities to inhibition by clorgyline (form A) or deprenyl (form B), respectively (Johnston, 1968). In both rat and monkey brains, MAO-A is found in non-serotonergic neurons and MAO-B is localized in 5-HT neurons as well as astrocytes (Westlund et al., 1985). We now report that intraneuronal and extraneuronal 5-HT levels are differentially changed in the presence of specific inhibitors of MAO. Deprenyl increased intracellular 5-HT levels (table 1) while clorgyline increased extracellular 5-HT levels (fig. 3). Furthermore, the effect of MDMA- and PCA-induced release of 5-HT from neurons pretreated with either clorgyline or deprenyl also differed (fig. 3). The combination of MDMA and clorgyline caused greater extracellular levels of 5-HT than either clorgyline or MDMA alone, but the levels were much less than the sum of those caused by MDMA and clorgyline separately. The lack of addition with MDMA/PCA and clorgyline may be partially explained by the reports that MDMA and PCA are specific MAO-A inhibitors (Fuller et al., 1975; Kokotos-Leonardi et al., 1992). On the other hand, deprenyl on its own did not increase extracellular 5-HT but did increase intracellular 5-HT and the combination of deprenyl and MDMA/PCA produced a potentiation of the extracellular 5-HT which was greater than the sum of their effects individually (fig. 3). These results indicate that the increase in extracellular 5-HT by MDMA/PCA is a combination of release of 5-HT by the transporter and inhibition of MAO-A activity. The extent of release of 5-HT depends on the availability of intracellular stores of 5-HT.

4.3. Reserpine inhibits vesicular 5-HT and reduces MDMA / PCA-induced release of 5-HT

We now provide evidence that these substituted amphetamines can indirectly affect the vesicular stores of 5-HT. Reserpine's action is to empty the intracellular vesicular store of 5-HT by inhibiting transport molecules in the vesicular membrane (Brodie et al., 1964). Reserpine was effective in our culture system in depleting 5-HT from the vesicular stores, since it reduced intracellular 5-HT levels by 78% after 48 h exposure compared to control condition. As a result of the reserpine-induced depletion, MDMA- and PCA-induced release of 5-HT was reduced by 20–50% (fig. 4). Furthermore, MDMA produced a greater reduction in the intracellular 5-HT levels in reserpine-free cultures (0.81 pmol/mg protein) than in reserpine-treated cultures (0.47 pmol/mg protein; table 1). Thus, in the absence of vesicular stores, there is less 5-HT available in the cell for MDMA-induced release.

The interactions between vesicular storage of 5-HT and cytoplasmic MAO-B activities on MDMA-sensitive intracellular 5-HT levels were further investigated. Deprenyl increased intracellular 5-HT levels in control cultures by 72% and in reserpinized cultures by 140%. However, the absolute increase of 5-HT levels by deprenyl was greater in control cultures than reserpine-treated cultures (table 1). This could be due to the integrity of the vesicular storage of 5-HT in control conditions. On the other hand, reserpine decreased 5-HT levels in control cultures by 67% and in deprenyl pretreated cultures by 55%. Again, the absolute decrease of 5-HT levels by reserpine was greater in cultures pretreated with deprenyl than in control cultures, which indicates an increase in the vesicular storage of 5-HT when MAO-B is inhibited.

Intracellular 5-HT was lower in cultures with both MDMA and deprenyl than that in cultures with deprenyl or MDMA alone (table 1). The possible explanation is that both cytoplasmic and vesicular stores were utilized in MDMA's action. It appears that chronic MDMA-induced release of 5-HT exits through the 5-HT transporter from cytoplasmic pools, which includes 5-HT drawn from vesicular stores. This idea is consistent with the mathematical analysis of CNS 5-HT intracellular distribution which concluded that the 5-HT contents of vesicular and cytoplasmic pools are in a dynamic equilibrium (Tracqui et al., 1983). The fluctuation of 5-HT levels in one compartment should lead to a corresponding adjustment on the another.

4.4. L-Type Ca^{2+} channels antagonist reduces MDMA / PCA-induced release

In acute studies, the release of 5-HT by MDMA is independent of Ca^{2+} (Nichols et al., 1982; Berger et

al., 1992). However, we and others have shown that Ca^{2+} was involved in MDMA's neuropathological action since both nimodipine (a specific L-type Ca^{2+} channel antagonist) and MK-801 (NMDA receptor-linked Ca^{2+} channel antagonist) attenuated the neuropathology of serotonergic neurons produced by MDMA (Azmitia et al., 1990; Azmitia, 1989; Farfel et al., 1992). We now report that in chronic studies involving 6 h of exposure, nimodipine (10^{-8} M) attenuated the MDMA- and PCA-induced release of 5-HT into the culture media by about 24 and 14% respectively (fig. 2). Meanwhile, acute studies involving [^{3}H]5-HT release from synaptosomes have shown that nimodipine modifies depolarization-induced (but not MDMA-induced) release of 5-HT (Kramer and Azmitia, unpublished observations). Therefore, it may be proposed that in chronic cultures, the integration of vesicular 5-HT into cytoplasmic pools requires intracellular Ca^{2+}.

In conclusion, our culture preparation showed that the both transporter-mediated release of 5-HT and inhibition of MAO-A activity by MDMA and PCA are involved in the increase of extracellular 5-HT. Furthermore, there appears to be a dynamic interaction between cytoplasmic and vesicular 5-HT stores in MDMA/PCA induced release of 5-HT. Evidence needs to be established for linking the absolute amounts of 5-HT released with neuropathology by MDMA and PCA. However, the evidence provided in this paper indicates that tissue culture serotonergic neurons show a number of the properties of mature neurons and can be used to analyze the molecular mechanisms underlying basic neurobiological phenomenon and the toxic events they may generate.

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