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Research report

Release of serotonin induced by 3,4-methylenedioxymethamphetamine (MDMA) and other substituted amphetamines in cultured fetal raphe neurons: further evidence for calcium-independent mechanisms of release

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Abstract

The substituted amphetamines 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), *p*-chloroamphetamine (PCA) and fenfluramine (FEN) all exert their effects by releasing serotonin (5-HT) from presynaptic nerve terminals. In the current study, we examined the ability of these agents to induce the release of 5-HT in cultured fetal raphe neurons. The data indicate that the rank order of release potencies for these agents was (±)PCA > (+)MDMA = (+)MDA = (±)FEN. Studies examining the role of calcium in 5-HT release demonstrate that preventing calcium influx with L- and N-type calcium channel blockers inhibits potassium-stimulated release of [^{3}H]5-HT but has no effect on release induced by the substituted amphetamines. Furthermore, omitting calcium from the extracellular media or depleting the vesicular pool of neurotransmitter with continual potassium stimulation did not affect the release of [^{3}H]5-HT induced by these compounds. Administration of fluoxetine prior to the substituted amphetamines significantly attenuated the releasing effects of these agents, while producing no effect on potassium-stimulated release. These results are consistent with the notion that the amphetamines induce release of cytoplasmic 5-HT via the plasma membrane transporter.

Keywords: Serotonin release; Culture; Fenfluramine; 3,4-Methylenedioxyamphetamine; *p*-Chloroamphetamine; Raphe; Nimodipine; Conotoxin; Fluoxetine

1. Introduction

The primary mechanism of action of amphetamine and its derivatives is to promote the release of endogenous monoamines from presynaptic nerve terminals. Some of these agents, namely 3,4-methylenedioxyamphetamine (MDA), *p*-chloroamphetamine (PCA), the anoretic agent fenfluramine (FEN) as well as the designer drug 3,4-methylenedioxymethamphetamine (MDMA, 'Ecstasy') are selective for the serotonergic system. These agents induce an acute release of serotonin (5-HT) both in vivo [18] and in vitro [27,36,42]. This release is associated with selective degeneration to the serotonergic system as evidenced by long term depletion of 5-HT and 5-HIAA levels [10,19,29,45,47] and decreased tryptophan hydroxylase activity [41,46,47]. There is also a loss of 5-HT immunoreactivity [1,11,32], morphological changes in fine 5-HT neurons arising from the dorsal raphe nucleus [33,35], and a decrease in 5-HT uptake sites [2,4,20,43,45,50]. These findings support the notion that these 5-HT releasing agents are selective neurotoxins for the serotonergic system.

Release of endogenous neurotransmitter may occur in two ways. One mechanism involves exocytotic or vesicular release, which is a process that is calcium-dependent and stimulated by potassium. The second mechanism is via the cytoplasmic release of neurotransmitter, a process that is calcium-independent, sodium-dependent and blocked by uptake inhibitors. The means by which amphetamine induces release has been postulated to occur via the second mechanism. One theory postulates that amphetamine enters the nerve terminal to displace neurotransmitter from vesicular stores by dissipating the pH gradient required for vesicular uptake of monoamines (weak base hypothesis; [48]). Increased concentrations of cytoplasmic pools may cause the normal direction of the plasma membrane transporter to reverse, resulting in cytoplasmic release of monoamines (for review, see [44]). However, there remains a paucity of definitive reports concerning the exact mechanism of action of substituted amphetamines. While

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some studies support the notion that MDMA, PCA, and FEN promote release of the cytoplasmic pools [6,38–40] others have suggested a concentration dependent, dual mechanism of release involving calcium [3,7,17,22,23]. For these reasons, the exact mechanism of action of these agents remains unclear.

In the present study, we examined the nature of release of [^{3}H]5-HT induced by (+)MDA, (+)MDMA, (±)PCA and (±)FEN using cultured fetal raphe neurons. The culture system affords advantages over other systems in that the releasing agents may be directly applied to intact, living neurons. The aim of this study was to characterize the mechanism of 5-HT release for these compounds in our tissue culture model.

2. Materials and methods

2.1. Preparation of dissociated cultures

The procedure for the preparation of dissociated cultures has been described previously [5]. Briefly, brains were obtained from fetal rats (E15) and placed in cold, sterile Earle's balanced salt solution. The rostral portion of the rhombencephalon extending from the rhombencephalic isthmus to the pontine flexure [30] was dissected and placed in sterile Dulbecco's modified eagle medium (DMEM) in 35 mm petri dishes. This region includes the anlages to the 5-HT-containing cell groups B7–9 which in the adult animal are located within the dorsal and medial raphe nucleus [12,30] as well as a population of tyrosine hydroxylase-immunoreactive neurons [30], presumed anlages to the DA cells in the adult dorsal raphe nucleus. Tissues were rinsed twice with sterile DMEM and mechanically dispersed. The cells were counted and plated at 7.5×10^5 cells per 16 mm well. All culture surfaces were precoated with poly-L-lysine (100 μg/ml). The plating media consisted of DMEM (4.5 g glucose/l, no pyruvate)/Ham's F12 (3:1) supplemented with 10% fetal calf serum, 10% horse serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 Units/ml penicillin. After 24 h, this media was removed and replaced with media containing 2% serum. The 2% serum media remained on the cultures for 24 h and then was replaced with serum free media. The serum free media used is a modification of that described by Bottenstein and Sato [9]. The cultures were maintained in an atmosphere of 5% CO_2/95% air at 37°C. Release experiments were performed on cells grown 8–10 days in culture. Previous studies by Hyde and Bennett [28] have provided evidence that the late gestational fetal rat transporter is functionally similar to the adult in terms of sodium-dependence as well as the affinity of the 5-HT transporter for uptake inhibitors. Other studies [16,52] have demonstrated the presence of 5-HT synthesis and metabolism in similar raphe cultures. Furthermore, these studies also indicated that there is an increase in 5-HT uptake capacity over time, which parallels the in vivo system. 5-HT neurons in culture possess a high affinity 5-HT uptake system, which is inhibited more than 95% by the specific 5-HT uptake inhibitor, fluoxetine (10 μM) [16,52]. These studies suggest that cultured fetal neurons possess aminergic properties which are similar to those found in adult 5-HT neurons.

2.2. Release protocol

Media was removed from the cultures and they were rinsed once with preincubation buffer (140 mM NaCl, 2.6 mM KCl, 1 mM Na_2HPO_4, 0.75 mM $MgCl_2$, 0.75 mM $CaCl_2$, 6 mg/ml glucose, 37°C, adjusted to pH 7.4). Incubation buffer (preincubation buffer with the addition of 100 μM pargyline, 600 μM ascorbate, 1 mg/ml BSA, 37°C, pH adjusted to 7.4) containing 50 nM [^{3}H]5-HT (NEN, 38–50 Ci/mmol) was added for 30 min in order to preload the cells with [^{3}H]5-HT. This loading period was terminated with the removal of the radioactive media and it was replaced with fresh incubation media lacking any radioactive material. The media was aspirated and replaced at five min intervals for a total of 20 min. After this wash period, fractions (0.5 ml) were collected every 5 min for a total of 11 fractions (55 min). Drugs or 30 mM KCl were added at fraction 7 (30 min into the paradigm) and removed at fraction 8. Scintillation fluid (Scintiverse BD, Fisher) was added to each fraction and monitored for radioactivity. Concentration curves for (+)MDMA, (+)MDA, (±)PCA, (±)FEN and (−)cocaine were obtained by examining the ability of these agents to promote [^{3}H]5-HT release into the media.

Experiments were conducted with calcium channel blockers, following the same protocol with the exception that the channel blockers (10.0 μM nifedipine, 1.0 μM conotoxin) were added to the media at the beginning of fraction 5 and remained in the media through fraction 7, when the releasing agents were administered. Similarly, studies utilizing fluoxetine were performed with the addition of 100 nM fluoxetine 10 min prior to the releasing agents. Other experiments involving the calcium-dependent nature of release were performed using calcium-free media ($CaCl_2$ removed and substituted with $MgCl_2$) containing 100 μM ethylene glycol tetraacetic acid (EGTA). In these studies, the normal incubation buffer was replaced with calcium-free media during the initial 20 min wash period, as were all subsequent fractions. Drugs or 30 mM KCl were added at fraction 7, which was chosen because basal [^{3}H]5-HT release was stable at this point. Additionally, experiments were performed to deplete the vesicular pool of [^{3}H]5-HT. This was accomplished by continual stimulation with 50 mM KCl (with adjustments made to maintain osmolality) and was present in all fractions preceding fraction 7. Either 30 mM KCl or the amphetamines were added at fraction 7 and [^{3}H]5-HT release determined after this persistent KCl depolarization. Stimulation at

fraction 7 with 30 mM KCl was necessary to assess the remaining vesicular [^{3}H]5-HT pool. Tissue content of [^{3}H]5-HT was assessed at the end of the experiment by lysing the cells overnight with 0.5 ml of 0.4 N $HClO_4$/100% ethanol (1:3). The lysate was added to scintillation cocktail and counted with the fractions on a Packard Scintillation counter (counting efficiency of 52%). Release was quantitated as fractional release and defined as:

$$\text{Fractional Release (\%)}: \quad \frac{\text{dpm in fraction 7}}{\text{dpm in all collected fractions plus dpm in tissue}} \times 100$$

All assays were performed in triplicate with less than 10% standard deviation between replicate samples. Statistical analyses were performed using Student's *t*-test or analysis of variance.

2.3. Materials

Pregnant Sprague-Dawley rats were purchased from Zivic-Miller (Zeleinople, PA, USA). [^{3}H]5-HT (38–50 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, MA, USA). Fluoxetine was obtained from Lilly (Indianapolis, IN), DL-*p*-Chloroamphetamine from Regis Chemical Company (Morton Grove, IL), and (±)fenfluramine from Sigma (St. Louis, MO). (+) 3,4-methylenedioxyamphetamine, (+)3,4-methylenedioxymethamphetamine and (−)cocaine were gifts from the Research Technology Branch of the National Institute on Drug Abuse.

3. Results

3.1. Comparison of [^{3}H]5-HT release from raphe cultures by (+)MDMA, (+)MDA, (±)PCA, (±)FEN and (−)cocaine

In initial studies, we examined the concentration-dependent nature of release induced by the substituted amphetamines. Results depicted in Fig. 1 demonstrate that (+)MDMA, (+)MDA, (±)PCA and (±)FEN all induce the release of [^{3}H]5-HT in a concentration-dependent manner. There is a remarkable difference in the potency of PCA versus that of the other substituted amphetamines to promote [^{3}H]5-HT release: (±)PCA is approximately ten times more potent at releasing [^{3}H]5-HT than the other compounds. These results differ somewhat from those of Schmidt et al. [42] in that they found MDMA and PCA to be equipotent releasers of [^{3}H]5-HT. Release of [^{3}H]5-HT by (−)cocaine, on the other hand, was marginal and not concentration-dependent. This is consistent with previous studies which indicate that cocaine is a pure uptake inhibitor [26,51] lacking releasing properties. Furthermore, this demonstrates that the increase in extracellular [^{3}H]5-

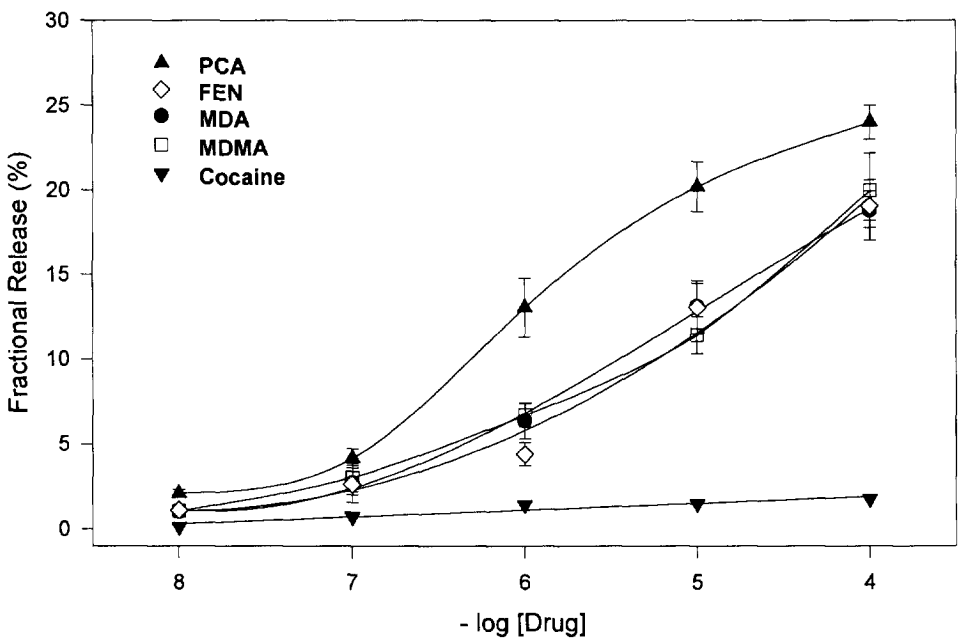

Fig. 1. The concentration-dependent nature of [^{3}H]5-HT release induced by (+)MDMA, (+)MDA, (±)PCA, (±)FEN and (−)cocaine. [^{3}H]5-HT release was assessed in the presence of various concentrations of the substituted amphetamines. Release was assessed over a 5 min period and expressed as fractional release. Significant increases in release were attained at concentrations of MDA, MDMA, and FEN greater than 1.0 μM, and at concentrations of PCA greater than 0.1 μM ($P < 0.05$) as determined by analysis of variance followed by Tukey's post-hoc analysis. (Asterisks were omitted for purpose of clarity.) The various concentrations of cocaine examined produced no significant increases in release. Values represent means ±S.E.M., $n = 6$ experiments performed in triplicate.

HT after administration of the substituted amphetamines is due to the releasing effects of these agents and not to their uptake inhibition properties.

3.2. Effect of calcium on the ability of (+)MDMA, (+)MDA, (±)PCA and (±)FEN to promote [^{3}H]5-HT release

Due to discrepancies in the literature regarding the calcium-dependent nature of the substituted amphetamines in releasing 5-HT, we decided to determine the role of Ca^{2+} in [^{3}H]5-HT release from raphe cultures (Fig. 2). The effects of the L-type Ca^{2+} channel blocker, nifedipine, as well as the N-type Ca^{2+} channel blocker omega-conotoxin are shown in Fig. 2. Nifedipine was found to prevent 90% of 30 mM K^+-stimulated [^{3}H]5-HT release at a concentration of 10 μM. Higher concentrations were not more effective and were found to cause cellular toxicity (unpublished observation). On the other hand, the presence of 10 μM nifedipine did not significantly attenuate the release induced by the substituted amphetamines. Similarly, the optimal concentration of omega-conotoxin was found to be 1.0 μM which resulted in a total abolishment of K^+ stimulated release of [^{3}H]5-HT (Fig. 3). However, administration of omega-conotoxin did not affect the release induced by the substituted amphetamines, indicating that neither of the major Ca^{2+} channel blockers (L- or N-type) alone or in conjunction (Fig. 2) significantly affected release stimulated by these agents.

Additional studies examined [^{3}H]5-HT release after removal of calcium from the media. Cells were exposed to a Ca^{2+}-free media (with the addition of 100 μM EGTA) before the drugs were administered and throughout the release period. Under these conditions, potassium-stimulated release (i.e. vesicular release) was significantly decreased from 12.88% fractional release under control conditions to 1.04% fractional release of [^{3}H]5-HT in the absence of calcium. Conversely, there was no significant difference in the release induced by (+)MDMA, (+)MDA, (±)PCA and (±)FEN in Ca^{2+}-free conditions versus release induced in control media (Fig. 4). There was a trend, however, for release induced by the substituted

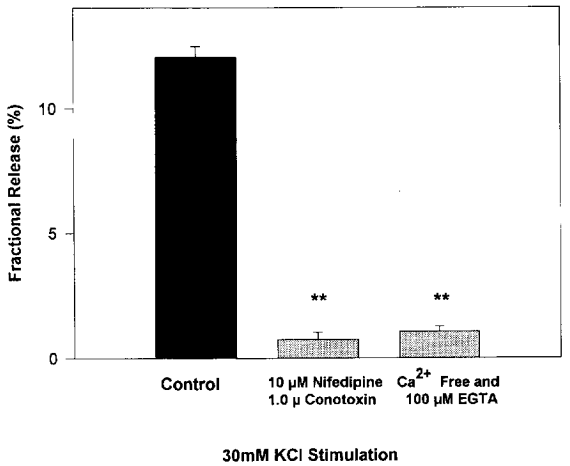

Fig. 3. The effect of calcium on K^+ stimulated release. [^{3}H]5-HT release in response to 30 mM KCl was examined in the presence and absence of calcium. Calcium was manipulated using either the 10 μM nifedipine and 1.0 μM conotoxin, or by omitting calcium from the media. Both conditions were significantly different from control (* $P < 0.01$). $n = 4$ experiments, each performed in triplicate.

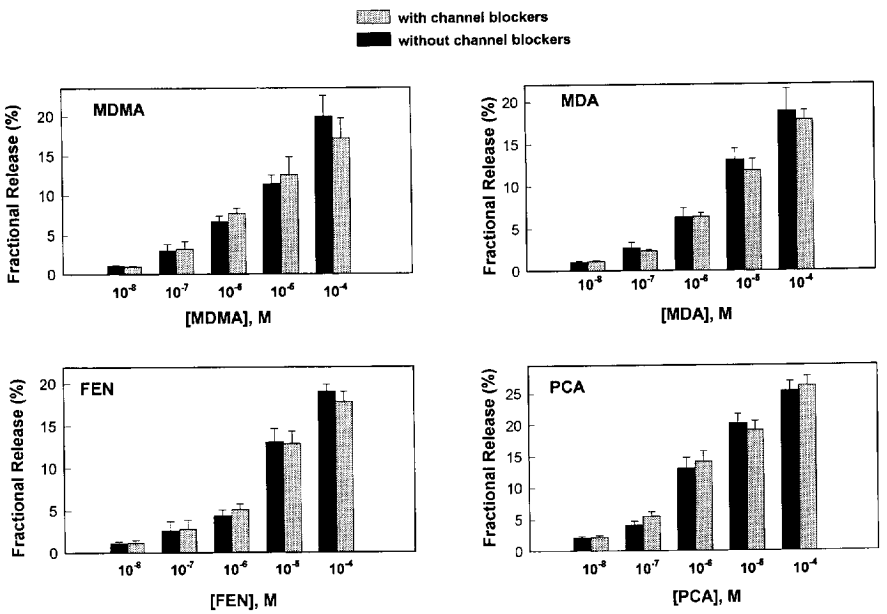

Fig. 2. The effect of calcium channel blockers on [^{3}H]5-HT release induced by (+)MDMA, (+)MDA, (±)PCA and (±)FEN. [^{3}H]5-HT release was assessed in the presence and absence of 10 μM nifedipine and 1.0 μM conotoxin. There was no significant difference in [^{3}H]5-HT release between control and calcium channel-treated cells at each concentration of the substituted amphetamine. $n = 4$ experiments, each performed in triplicate.

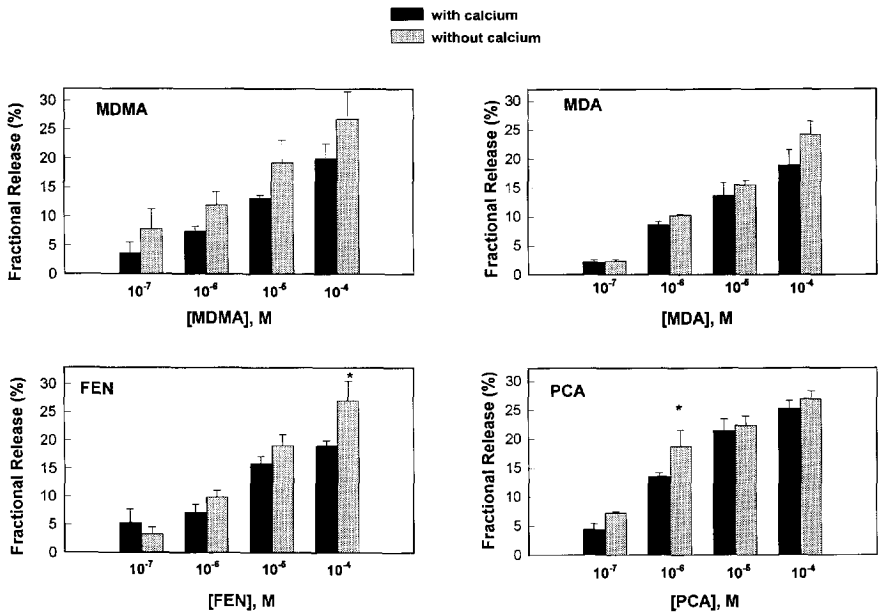

Fig. 4. The effect of calcium on [^{3}H]5-HT release induced by (+)MDMA, (+)MDA, (±)PCA and (±)FEN. [^{3}H]5-HT release induced by various concentrations of (+)MDMA, (+)MDA, (±)PCA and (±)FEN was assessed in the presence and absence of calcium in the media. Significance between the treated and control groups at the concentration of substituted amphetamine is indicated (* $P < 0.05$). $n = 4$ experiments, each performed in triplicate.

amphetamines to be greater in the absence of calcium and this was observed consistently for each drug.

3.3. Effect of depleting the 5-HT vesicular pool on release promoted by KCl or the substituted amphetamines

In a separate set of experiments, a depolarizing media (50 mM KCl) was utilized to promote the depletion of the vesicular pool (including both endogenous 5-HT and [^{3}H]5-HT). This high KCl media was added to the cultures throughout all the initial equilibration periods and removed at the release period at which time either 30 mM KCl or the amphetamines were added. Release stimulated by 30 mM KCl was significantly reduced from 12% to 3%, indicative of a depletion of the vesicular pool (Fig. 5). On the other hand, there was no significant attenuation of

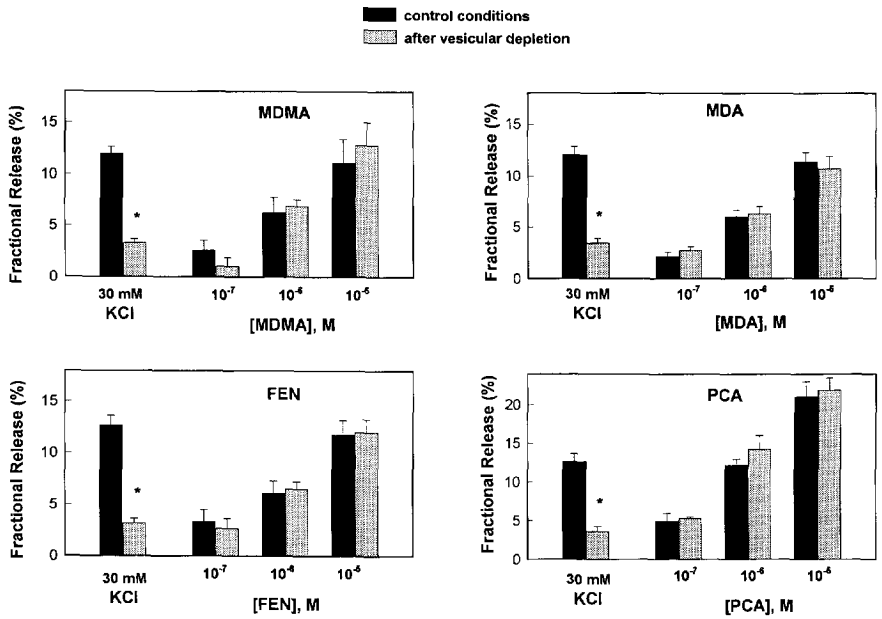

Fig. 5. The effect of vesicular pool depletion on [^{3}H]5-HT release induced by(+)MDMA, (+)MDA, (±)PCA and (±)FEN. The vesicular pool was depleted by continual stimulation with 50 mM K^+. The depolarizing media was removed and (+)MDMA, (+)MDA, (±)PCA, (±)FEN or 30 mM KCl subsequently administered. While the K^+ stimulated release was significantly decreased (* $P < 0.05$), there was no significant difference between the vesicular-depleted and control groups at each concentration of substituted amphetamine. $n = 4$ experiments, each performed in triplicate.

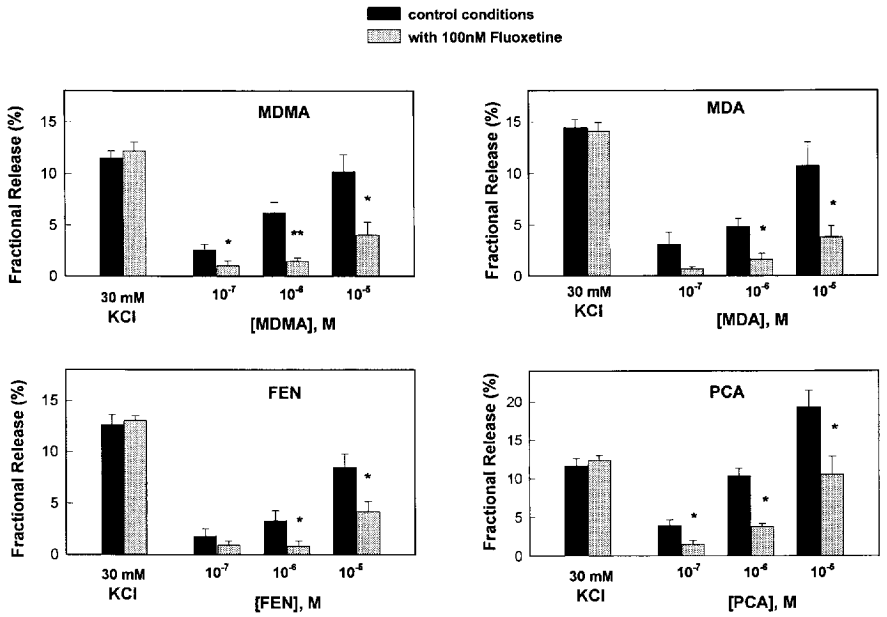

Fig. 6. The effect of fluoxetine pretreatment on [^{3}H]5-HT release induced by (+)MDMA, (+)MDA, (±)PCA and (±)FEN. When 100 nM fluoxetine was administered 10 min prior to the substituted amphetamines, there was a significant decrease in [^{3}H]5-HT release (* $P < 0.05$; ** $P < 0.01$) when compared to controls. K^+-stimulated release was not attenuated in the presence of fluoxetine. $n = 4$ experiments, each performed in triplicate.

release produced by (+)MDMA, (+)MDA, (±)PCA or (±)FEN.

3.4. Effect of the uptake inhibitor, fluoxetine, on [^{3}H]5-HT release by (+)MDMA, (+)MDA, (±)PCA and (±)FEN

Fluoxetine, a specific 5-HT uptake inhibitor, was added to prevent the transport of [^{3}H]5-HT from the cytoplasm via the membrane carrier to the outside of the cell. Administration of 100 nM fluoxetine 10 min prior to the substituted amphetamines attenuated the fractional release of [^{3}H]5-HT for the substituted amphetamines while there was no significant effect on KCl-stimulated release (Fig. 6).

4. Discussion

The substituted amphetamines (+)MDMA, (+)MDA, (±)PCA and (±)FEN are indirect sympathomimetics known to exert their effects on neurons primarily via the release of presynaptic 5-HT [15,18,27,36]. In the present study, two main questions were addressed: in cultured fetal raphe neurons, (1) what is the nature of release of [^{3}H]5-HT by these agents (calcium-dependent or calcium-independent) and (2) do these four selective 5-HT releasing agents exert their effects via a similar mechanism. The results indicate that these substituted amphetamines release [^{3}H]5-HT in cultured neurons through a similar mechanism. This process does not occur via a calcium-dependent, exocytotic release of the vesicular pool, but instead is likely mediated via the fluoxetine-sensitive, cytoplasmic pool of neurotransmitter.

The present study utilized cultured fetal neurons to assess the release of [^{3}H]5-HT induced by these various release-promoting agents. The ability to study neurons from a distinct brain region in culture offers many advantages over other in vitro methods. The culture system provides a controlled environment where drugs may be directly applied and pharmacological properties of a defined population of neurons can be examined without interferences from other systems. The mechanism by which these agents promote the release of [^{3}H]5-HT in culture remains controversial since there is only one other report [24] that currently has addressed this issue. In the present study, the neurons were preloaded with [^{3}H]5-HT prior to the release portion of the experiment. Previous studies have demonstrated that release of preloaded [^{3}H]dopamine closely resembled the release of newly synthesized dopamine from [^{3}H]tyrosine [14]. Thus, the current method may parallel that occurring in vivo in which the newly synthesized pool of neurotransmitter is preferentially released [49]. In addition, stimulation with 30 mM K^+, which would promote the release of vesicular stores of [^{3}H]5-HT, was found to release approximately 12–15% of the total [^{3}H]5-HT present in the tissue. For these reasons, we feel that the methods employed in the present experiments were not predisposed to biasing one intraneuronal pool of neurotransmitter.

Our first series of experiments examined the ability of (+)MDMA, (+)MDA, (±)PCA and (±)FEN to induce release in a concentration-dependent manner as well as

determine the relative potency of these agents to each other. Confirming previously reported studies, our results demonstrate that the releasing effects of these agents is concentration-dependent [6,22,34]. However, (±)PCA was approximately 10-fold more potent than the other agents. This is in disagreement with studies by Berger et al. who demonstrated in synaptosomal preparations that (±)PCA was equipotent to (+)FEN [6], as well as studies by McKenna et al. who suggest that (+)MDA is equipotent to (±)PCA [34]. Differential rank order potencies for (+)MDA and (±)FEN may be attributed to differences in methodologies since their studies involved the use of synaptosomes prepared from different brain regions. In addition to the substituted amphetamines, we also examined the ability of (−)cocaine to induce the release of [^{3}H]5-HT. The increase in extracellular [^{3}H]5-HT induced by (−)cocaine was marginal and not concentration-dependent. This finding is consistent with the notion that cocaine is a pure uptake inhibitor [26,51]. Furthermore, it demonstrates that the significant increase in extracellular [^{3}H]5-HT that is seen following administration of substituted amphetamines is not attributed to the uptake inhibition properties of these agents, but is due to their release-promoting effects.

Further studies were designed to examine the necessity for calcium in the release of [^{3}H]5-HT induced by the serotonergic selective substituted amphetamines. The influx of calcium following depolarization of a neuron is responsible for triggering the exocytosis of synaptic vesicles and the subsequent release of neurotransmitters [37]. Thus, in the first series of experiments, we blocked the influx of calcium into the neurons using the calcium channel blockers nifedipine (10 μM) and conotoxin (1.0 μM). Under these conditions, we were able to abolish the potassium-stimulated (vesicular) release, while there was no effect on the substituted amphetamine-induced release. This result is in apparent disagreement with the findings of Gu and Azmitia [24] which demonstrated that nimodipine significantly attenuated the release of 5-HT induced by (+)MDMA after a prior 6-h exposure to this agent. Our results are also in partial disagreement with those of Gobbi et al. [23] in which conotoxin (0.5 μM) was found to significantly decrease the release of 5-HT induced by 0.5 μM fenfluramine. To further confirm our results, we performed similar experiments examining the release of [^{3}H]5-HT by the substituted amphetamines after completely omitting calcium from the media. We found, however, that we were unable to completely attenuate the potassium-stimulated release unless we also included 100 μM EGTA, which is consistent with the findings of Berger et al. [6]. While these conditions attenuated potassium-stimulated release, the [^{3}H]5-HT release induced by the amphetamines was not reduced and even revealed a trend towards increased release. This trend may be due to the accumulation of sodium ions in the interior of the cell as it has previously been shown that EGTA has an inhibitory effect on the Na^+,K^+-ATPase [8,25]. The plasma membrane monoamine transporter is thought to reverse the direction of neurotransmitter flow if the sodium gradient is altered, so the additional release or additive effect seen under calcium-free/EGTA conditions lends further credence to postulate that these substituted amphetamines promote release through cytoplasmic-mediated mechanisms.

The role of the vesicular pool in the release of [^{3}H]5-HT promoted by the substituted amphetamines was examined by an additional experiment. In these studies, the vesicular pool of neurotransmitter was gradually depleted with continual potassium stimulation prior to the administration of (+)MDMA, (+)MDA, (±)PCA and (±)FEN. This method was employed as an alternative to the traditional method utilizing reserpine because studies have demonstrated that reserpine disrupts the synthesis of catecholamines, reducing the synthesis of new amines which could feasibly alter the ratio of the pools of neurotransmitter and thus the labeled to unlabeled 5-HT in our experiments [13]. In our study, the vesicular pool of 5-HT was depleted as shown by the decrement of release following subsequent potassium stimulation (75% reduction in release). On the other hand, the ability of the substituted amphetamines to release [^{3}H]5-HT was unaltered. Overall, these experiments in which we examined the calcium-dependent nature of release are in discrepancy with the concentration-dependent mechanism of fenfluramine release proposed by Gobbi and Bonanno [7,23] and provide further in vitro evidence against a calcium-dependent mechanism of release of [^{3}H]5-HT induced by (+)MDMA, (+)MDA, (±)PCA and (±)FEN.

There are several approaches that are acceptable methods to study the cytoplasmic release of 5-HT. All of these methods employ manipulation of the plasma membrane transporter. One method reverses the direction of the transporter by altering the sodium gradient either by ouabain or replacing extracellular sodium with lithium. The other approach involves blocking cytoplasmic release by blocking the transporter with an uptake inhibitor such as fluoxetine or paroxetine (which may effectively 'lock' the transporter in place). We attempted to alter the sodium gradient in our system by replacing the extracellular sodium with lithium. We were able to release [^{3}H]5-HT only when all of the extracellular sodium was replaced with lithium. Under these conditions, however, the release plateaued at maximal levels such that it was impossible to examine additive effects of administering the substituted amphetamines at the same time (unpublished results). We also found that the release induced by ouabain was sufficient, but too erratic for detailed analyses (unpublished results). We were successful in attenuating release by 'blocking' the plasma membrane 5-HT transporter with fluoxetine, a compound that binds with high affinity to the transporter, but does not itself appear to be transported into the cell [21]. Although the serotonin transporter normally removes

the neurotransmitter from the synapse and returns it to the presynaptic terminal, the serotonin transporter on the plasma membrane may also work in the opposite direction to mediate the release of cytoplasmic pools of 5-HT [31,40]. If the substituted amphetamines exert their effects by releasing cytoplasmic pools of 5-HT, then 'blocking' the transporter with fluoxetine should attenuate the release. Results from our study showed a significant attenuation of release of [^{3}H]5-HT following administration of 100 nM fluoxetine for all of the amphetamines examined. There was a small discrepancy between the sensitivity of (±)PCA versus the other substituted amphetamines when administered with fluoxetine that may be due to the fact that (±)PCA is tenfold more potent a releasing agent when compared to (+)MDMA, (+)MDA and (±)FEN. These results suggest that the substituted amphetamines induce release via a cytoplasmically-mediated process and are in agreement with previous studies utilizing different in vitro protocols [38–40,42]. The data from the present study using primary cultures suggest that the release of [^{3}H]5-HT induced by (+)MDMA, (+)MDA, (±)PCA and (±)FEN occurs through a similar mechanism which is not associated with a calcium-dependent mechanism, but is most likely mediated via cytoplasmic pools through the reversal of the plasma membrane transporter.

Acknowledgements

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