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Neonatal Citalopram Treatment Inhibits the 5-HT Depleting Effects of MDMA Exposure in Rats

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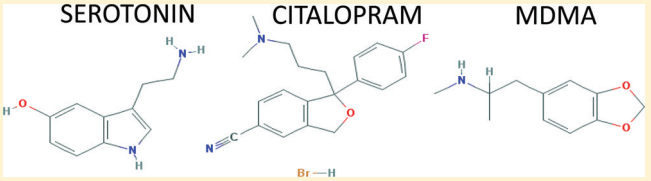
Supporting Information

ABSTRACT: Neonatal exposure to 3,4-methylenedioxymethamphetamine (MDMA) produces long-term learning and memory deficits and increased anxiety-like behavior. The mechanism underlying these behavioral changes is unknown, but we hypothesized that it involves perturbations to the serotonergic system as this is the principal mode of action of MDMA in the adult brain. During development, 5-HT is a neurotrophic factor involved in neurogenesis, synaptogenesis, migration, and target region specification. We have previously shown that MDMA exposure (4 × 10 mg/kg/day) from postnatal day (P)11−20 (analogous to human third trimester exposure) induces ∼50% decreases in hippocampal 5-HT throughout treatment. To determine whether MDMA-induced 5-HT changes are determinative, we tested if these changes could be prevented by treatment with a selective serotonin reuptake inhibitor (citalopram: CIT). In a series of experiments, we evaluated the effects of different doses and dose regimens of CIT on MDMA-induced 5-HT depletions in three brain regions (hippocampus, entorhinal cortex, and neostriatum) at three time points (P12, P16, P21) during the treatment interval (P11−20) known to induce behavioral alterations when animals are tested as adults. We found that 5 mg/kg CIT administered twice daily significantly attenuated MDMA-induced 5-HT depletions in all three regions at all three ages but that the protection was not complete at all ages. Striatal dopamine was unaffected. We also found increases in hippocampal NGF and plasma corticosterone following MDMA treatment on P16 and P21, respectively. No changes in BDNF were observed. CIT treatment may be a useful means of interfering with MDMA-induced 5-HT reductions and thus permit tests of the hypothesis that the drug's cognitive and/or anxiety effects are mediated through early disruptions to 5-HT dependent developmental processes.



KEYWORDS: *Serotonin, dopamine, development, ecstasy, corticosterone, citalopram*

Over the past decade 3,4-methylenedioxymethamphetamine (MDMA) has become a popular drug of abuse especially with adolescents and younger adults of whom 3−12% report use within the past year.[28] Adult exposure to MDMA has been shown to disrupt verbal recall and spatial associative learning.[84] Similarly, adult rats that were administered a serotonin (5-HT) depleting regimen of MDMA demonstrated reference memory deficits in the Morris water maze (MWM) and learning disruptions in the Cincinnati water maze (CWM) when tested under low light conditions.[1,70] Congruent with adult human MDMA abuse, women who abuse MDMA and are pregnant expose their fetus to MDMA.[24,46] The effects of developmental exposure have not been well investigated clinically, although there are reports of increased incidences of cardiac malformations and club-foot.[45,46,71] No prospective studies exist that examine the long-term cognitive and neurological outcomes of children exposed to MDMA in utero. The neonatal rat has been used to model human second through third trimester brain development.[10] MDMA exposure from postnatal day (P) 11−20 causes deficits in spatial and egocentric learning and increases in anxiety when the offspring are tested as adults.[66,72−74,79] The mechanism(s) by which developmental MDMA produces these long-term alterations is unknown.

MDMA binds to serotonin transporter (SERT),[57] resulting in (1) inhibited 5-HT reuptake,[27] (2) MDMA uptake into the cytosol,[56] (3) reversal of SERT flux causing 5-HT overflow,[2] (4) redistribution of SERT,[31,32] and (5) interference with VMAT2 transport of 5-HT in vesicles.[5] At sufficient doses of MDMA, prolonged 5-HT release followed by long-term 5-HT depletion in adults has been reported (reviewed in ref 21) and we have shown decreases in hippocampal and neostriatal 5-HT

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and 5-hydroxyindolacetic acid (5-HIAA) levels throughout the dosing period known to produce long-term cognitive deficits[63,64,80] when administered during development. Neurotransmitter depletions during development can be detrimental since 5-HT acts as a neurotrophic factor that supports the development of 5-HT neurons and neurons in various target regions.[76] Following developmental PCA- or PCPA-induced 5-HT depletions, delays are seen in neuronal proliferation and migration, decreases are observed in neuronal spine density, and deficits are seen in spatial learning in the radial-arm maze.[35,36,44,76,83]

Early MDMA-induced 5-HT reductions may contribute to the learning and memory deficits induced by developmental MDMA exposure, although a direct test of this hypothesis is lacking. The purpose of this study was to determine if inhibiting SERT with a selective serotonin reuptake inhibitor (SSRI) would attenuate MDMA-induced 5-HT reductions in brain regions innervated by the serotonergic system and known to be involved in learning and memory. Previous studies have shown that fluoxetine pre- or coadministration with MDMA prevents MDMA-induced 5-HT and 5-HIAA reductions in striatum and hippocampus[43,60] and citalopram (CIT) pretreatment prior to MDMA administration has been shown to block MDMA-induced reductions in exploration and aggression in adult animals.[54] For the current study, citalopram was chosen because it is more selective for SERT than fluoxetine[59] and is less potent at inhibiting cytochrome P450s that are important in MDMA metabolism.[15,23]

In order to identify a CIT dose that would be most effective at blocking or attenuating MDMA-induced 5-HT reductions, we tested several regimens in combination with MDMA using a single day or multiple days of exposure that included P11, P11−15, and P11−20. We found that two doses per day of 5 mg/kg of CIT were effective at interfering with MDMA-induced 5-HT reductions after P11−20 exposure. To confirm this effect at earlier ages, we also tested the effect of CIT treatment following P11 and P11−15 MDMA exposure.

The brain regions assessed included the entorhinal cortex (EC), hippocampus, and neostriatum (caudate and putamen).[69] The EC is innervated by the serotonergic system from the raphe nuclei, is the main input pathway to the hippocampus, and has a high density of 5-HT receptors.[4,52,53] The EC plays a role in allocentric and egocentric learning,[16,47,61,81] both of which are altered by developmental (P11−20) MDMA exposure. Importantly, 5-HT has been shown to suppress excitatory synaptic transmission in the superficial layers further supporting the involvement of EC 5-HT levels in learning.[65] Spatial learning in the MWM is known to be hippocampally dependent[50] and the hippocampus is heavily innervated by the serotonergic system.[25] Further, hippocampal granular cells are proliferating at a high rate during human third trimester and during the rodent neonatal period assessed herein.[3,11] The striatum receives input from raphe 5-HT axons, and lesion studies have shown that the striatum is important for egocentric learning,[8,12] and drug induced 5-HT decreases in the striatum correlate with decreased egocentric learning in adult rats.[13]

MDMA also increases corticosterone[63,64,80] which can interfere with hippocampal neuronal proliferation,[17,39,82] and some neurotrophins[33] that are important neuronal maintenance factors involved in learning and memory.[9,30,38,49] The neurotrophins, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), have been shown to be influenced by stress/corticosterone levels and changes in 5-HT.[55,62,68,85] CIT has also been shown to alter these neurotrophins in adults.[22,26,29,58] To potentially identify additional factors that could alter long-term cognition in addition to the proposed developmental 5-HT reductions, we assessed corticosterone in plasma, and NGF and BDNF levels in the hippocampus and neostriatum. To determine if early CIT treatment and/or MDMA had lasting effects, we assessed 5-HT and dopamine (DA) at P60, an age at which we previously found learning and memory impairments after P11−20 MDMA exposure.[73,74,79]

RESULTS AND DISCUSSION

Experiment 1. The first experiment determined whether one CIT treatment (1.25, 2.5, 5, or 10 mg/kg) attenuates hippocampal 5-HT reductions induced by P11 MDMA exposure (10 mg/kg every 2 h for 4 doses, see Figure 1 for dosing schedule). For this, the effects of CIT treatment were examined 24 h after MDMA treatment on P11 (P12). P11 was chosen since it is the first day of the 10 day MDMA treatment regimen that induces behavioral changes, and the 24 h time-point was used because the largest MDMA-induced 5-HT reductions occur at this interval.[80] Hippocampal 5-HT levels were significantly decreased on P12 in the SAL+MDMA-treated group compared with the SAL+SAL group as predicted (Figure 2a). All CIT treatment groups attenuated MDMA-induced hippocampal 5-HT reductions compared with the SAL +SAL group. No dose level of CIT+SAL altered hippocampal 5-HT. All CIT+MDMA treatments had significantly increased hippocampal 5-HT levels compared to the SAL+MDMA-treated group except the 1.25 CIT+MDMA-treated animals.

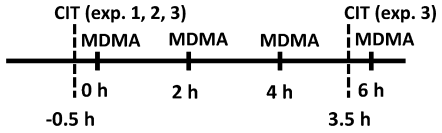

Figure 1. Schematic of dosing schedule.

Experiment 2. The 2.5 mg/kg CIT dose was then used to assess its influence on MDMA-induced 5-HT reductions after 10 days of CIT+MDMA treatment (P11−20). We did not use the 10 mg/kg dose because previously published data show that P8−21 CIT treatment causes behavioral alterations.[40−42] 2.5 CIT treatment on P11−20 had only a minor attenuating effect on decreased hippocampal 5-HT from MDMA exposure when examined on P21, although this treatment was significantly increased compared to SAL+MDMA-treated animals (Figure 2b).

Experiment 3. The P21 outcome from experiment 2 suggested that with longer exposures the adequacy of a single CIT treatment was insufficient. This may have occurred because the four MDMA doses out-competed the single dose of CIT for SERT binding sites. We have previously shown that MDMA in neonates has a half-life of approximately 4 h,[77] and given that the half-life of CIT is 3 h in adult rats[48] (there are no comparable data in neonatal rats), additional doses of CIT might provide better inhibition. Therefore, we tested whether two treatment doses of CIT were more effective. Hence, the following groups were prepared: 1 × 5, 2 × 5, 1 × 10, and 2 × 10 mg/kg CIT given 0.5 h prior to the first and fourth dose of 10 mg/kg MDMA (P11−20). We administered the second CIT dose before the fourth MDMA dose instead of earlier in

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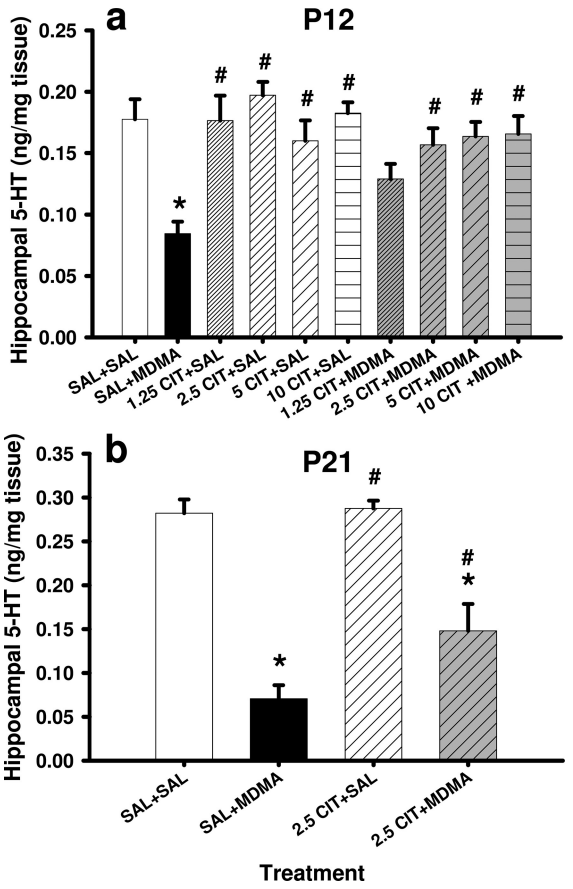

Figure 2. Hippocampal 5-HT levels following (a) P11 and (b) P11−20 treatment and measured 24 h later at P12 and P21, respectively. (a) On P12, the SAL+MDMA group showed a >50% reduction in 5-HT levels. No significant changes were observed in any of the CIT+SAL groups. The three higher CIT+MDMA groups (CIT 2.5, 5, 10) showed nearly full protection from MDMA exposure, while the 1.25 CIT+MDMA showed a trend toward protection being midway between the SAL+MDMA and SAL+SAL groups. (b) Following P11−20 exposure, the SAL+MDMA-treated group showed even larger reductions of ∼70%. The CIT+MDMA group (CIT 2.5) showed no effects. The CIT+MDMA group showed an attenuation of the effects compared to SAL+MDMA but were still markedly reduced compared with the SAL+SAL group. (a) Main effect of treatment ($F(9,63) = 6.86$, $p < 0.0001$); $n = 9$ for 5 CIT+MDMA and 1.25 CIT+MDMA, $n = 7$ for 5 CIT+SAL and 1.25 CIT+MDMA; $n = 8$ for all other treatment groups. (b) Main effect of treatment ($F(3,32) = 49.42$, $p < 0.0001$); $n = 5$/treatment. *$p < 0.05$ different from SAL+SAL treatment; #$p < 0.05$ different from SAL+MDMA treatment.

the dosing paradigm to ensure that CIT would still inhibit MDMA binding to SERT following the fourth dose of MDMA.

The 2 × 5 and 1 × 10 CIT+MDMA treatment groups were the most effective at interfering with MDMA-induced 5-HT reductions on P21 (Figure 3). Although the 2 × 5 CIT +MDMA group was not as protected as the 1 × 10 CIT +MDMA group, for the reason noted above we were reluctant to use the single 10 mg/kg CIT dose. The 2 × 10 CIT+MDMA group showed no protection and in fact showed 5-HT reductions similar to that of the SAL+MDMA group. This paradoxical effect ruled this group out from further consideration. This effect may have been the result of saturation of monoamine oxidase (MAO), which is inhibited by MDMA.[37] MAO is responsible for the metabolism of CIT and 5-HT. Hence, too much CIT may contribute to the 5-HT

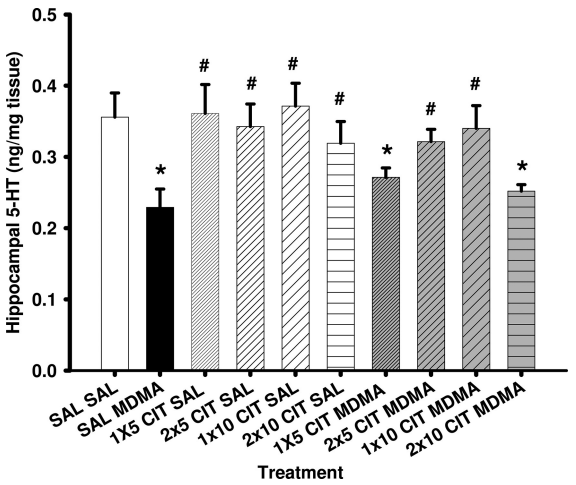

Figure 3. P21 hippocampal 5-HT. SAL+MDMA significantly reduced 5-HT levels. None of the CIT+SAL groups showed changes compared with the SAL+SAL group. The 1 × 5 CIT+MDMA and 2 × 10 CIT +MDMA groups where higher than the SAL+MDMA group but significantly below the SAL+SAL group. However, the 2 × 5 and 1 × 10 CIT-MDMA groups were significantly higher than the SAL +MDMA group and not different from the SAL+SAL group. Main effect of treatment ($F(9,53) = 6.8$, $p < 0.0001$); $n = 7$/treatment. *$p < 0.05$ different from SAL+SAL; #$p < 0.05$ different from SAL+MDMA.

reduction seen in this group,[6,34] since MAO saturation may result in an increased outflow of 5-HT to a degree that inhibition of tryptophan hydroxylase occurs,[41] resulting in reduced 5-HT synthesis.

The 2 × 5 CIT regimen therefore appeared the optimal regimen to test whether it could attenuate MDMA-induced reductions in 5-HT. Litters were prepared and 5-HT, DA (where applicable), their metabolites, hippocampal and neostriatal NGF and BDNF, and corticosterone in plasma were examined. The groups were SAL+SAL, SAL+MDMA, CIT +SAL, and CIT+MDMA with treatment on P11, P11−15, or P11−20 and assayed on P12, P16, or P21, respectively.

Hippocampus. As expected 5-HT levels were significantly decreased in the SAL+MDMA group compared with the SAL +SAL group at all ages (Figure 4a). The CIT+SAL group was expected to have no effect on 5-HT, but in fact it partially reduced levels on P12 compared to the SAL-SAL group. This effect was not seen at P16 or P21. On P12 and P16, the CIT +MDMA group interfered with MDMA-induced 5-HT reductions compared with the SAL+SAL group but the effect was incomplete. The CIT+MDMA-treated animals had significantly higher 5-HT levels than the SAL+MDMA group at all three ages but were also significantly below the SAL+SAL group at P12 and P16. Only at P21 was the CIT+MDMA group not different from the SAL+SAL group (Figure 4a−c). Hippocampal 5-HIAA was significantly decreased and the 5-HIAA/5-HT ratio was increased at most time points following SAL+MDMA. CIT+SAL produced a decrease in 5-HIAA on P12 and an increase on P21. The 5-HIAA/5-HT ratio was increased on P16 and P21 following CIT+SAL treatment. CIT +MDMA did not attenuate hippocampal 5-HIAA decreases or the 5-HIAA/5-HT ratio compared to SAL+MDMA treatment on P12. However, on P16 and 21, the combination prevented any changes in metabolite and ratio compared to SAL+SAL treatment. For 5-HIAA and 5-HT utilization effects, see Supporting Information Table S1.

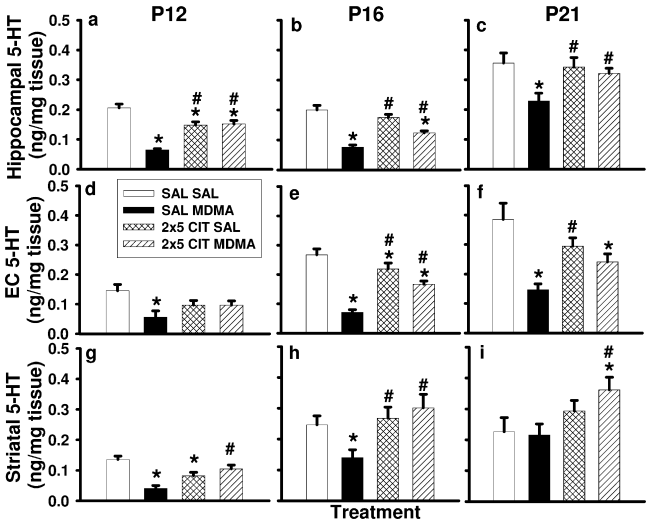

Figure 4. P12, 16, and P21 5-HT levels: P12 groups were treated on P11, P16 groups were treated on P11−15, and P21 groups were treated on P11−20. (a) Main effect of treatment ($F(3,21) = 39.03$, $p < 0.0001$); (b) main effect of treatment ($F(3,21) = 60.14$, $p < 0.0001$); (c) main effect of treatment ($F(9,53) = 6.80$, $p < 0.0001$); (d) main effect of treatment ($F(3,32) = 5.02$, $p < 0.009$); (e) main effect of treatment ($F(3,21) = 44.2$, $p < 0.0001$); (f) main effect of treatment ($F(3,18) = 10.34$, $p < 0.0003$); (g) main effect of treatment ($F(3,32) = 19.97$, $p < 0.0001$); (h) main effect of treatment ($F(3,21) = 8.03$, $p < 0.0009$); (i) main effect of treatment ($F(3,18) = 3.50$, $p < 0.04$); n = 8/treatment/age/brain region. *$p < 0.05$ different from SAL+SAL; #$p < 0.05$ different from SAL+MDMA.

Entorhinal Cortex. This is the first study to examine MDMA-induced 5-HT depletions in the EC during development. As can be seen in Figure 4d−f, developmental MDMA exposure induced greater than 50% reductions at all three ages. CIT+SAL exposure did not alter 5-HT on P12 or P21; however, on P16, EC 5-HT levels were slightly reduced compared to the SAL+SAL group. This effect was not as severe as seen in the SAL+MDMA group. In this region, CIT treatment completely attenuated MDMA-induced 5-HT depletions only on P12. Following P11−15 and P11−20 exposure, CIT+MDMA altered levels of 5-HT were significantly decreased compared to the SAL+SAL group, but importantly they were significantly higher than the SAL +MDMA group, indicating a partial protection effect. 5-HIAA in the EC was decreased in all groups at all ages with the exception of the CIT+SAL group which was unchanged on P16 and increased on P21 compared to the SAL+SAL group. The 5-HIAA/5-HT ratio in the EC was increased in the SAL+MDMA group on P12 and P16 and decreased on P21 compared to SAL +SAL-treated animals. CIT+SAL only increased the ratio on P21. The combination prevented changes in the ratio compared to SAL+SAL-treated animals on P12 and P16. For 5-HIAA and 5-HT utilization effects, see Supporting Information Table S1.

Neostriatum. In the neostriatum, the effect of SAL+MDMA was as predicted; that is, at P12 and P16, 5-HT levels were significantly decreased, but by P21 levels in this group did not differ from the SAL+SAL group (Figure 4g−i). The CIT+SAL group did not alter 5-HT levels on P16 or P21. However, 5-HT levels were decreased on P12 as was observed in the hippocampus. In terms of providing protection against MDMA-induced 5-HT reductions, the CIT+MDMA group showed significant protection. CIT+MDMA attenuated 5-HT reductions to a level not significantly below that of the SAL +SAL group at all ages. Unexpectedly, however, this group showed significantly higher 5-HT levels at P21 compared with the SAL+SAL group. Neostriatal 5-HIAA and 5-HT utilization effects followed a similar pattern as in the hippocampus; see Supporting Information Table S1.

We previously showed that MDMA does not alter neostriatal DA levels on P12, 16, or 21.[63,64,80] The results of the current experiment confirm this finding. As with the SAL+MDMA-treated animals, neither CIT+SAL nor CIT+MDMA exposure altered neostriatal DA levels (see Supporting Information Table S1). Although neostriatal DA concentrations were not altered, there is evidence that the dopaminergic system is affected by MDMA exposure. In both MDMA groups, dihydroxyphenylacetic acid (DOPAC) levels and DOPAC/DA ratios were lower on P12 and higher on P21 compared to SAL-treated and SAL +CIT groups (see Supporting Information Table S1). This suggests that there is a differential effect of acute versus chronic MDMA exposure or the degree of maturation plays a role in the response of the DA system to developmental MDMA. It is important to note that changes in DOPAC levels and DOPAC/DA ratios were not long lasting (see below).

Adult Effects. In order to determine the long-term effects of 2 × 5 CIT prior to the 10 mg/kg MDMA treatment 4 times per day from P11−20, separate groups were treated and assayed on P60. In the hippocampus (Figure 5a), the SAL+MDMA group showed significant reductions in 5-HT levels. Unexpectedly, the CIT+SAL group also showed reductions.

In the EC, no lasting effects of MDMA, CIT, or the combination were found; that is, there was no residual 5-HT reduction in the SAL+MDMA group, no effect in the CIT+SAL group, and no combined effect in the CIT+MDMA group (Figure 5b).

Similarly, there were no significant group differences in neostriatal 5-HT, although a trend toward reduced levels in the SAL+MDMA group compared to the SAL+SAL group occurred, but because of greater variation at this age this trend was not statistically significant (Figure 5c).

There were no significant group differences in 5-HIAA, DA, DOPAC, or 5-HT or DA utilization among the groups in the hippocampus, EC, or neostriatum; see Supporting Information Table S2.

It is interesting to note that there appears to be region and age specific effects of the dosing paradigm including the lack of 5-HT changes following MDMA treatment in the neostriatum at P21 and in the EC at P12, and sporadic 5-HT changes following CIT exposure. This may be attributed to changes in monoamine innervations including differentiation, migration, and synapse formation or alterations in the rate of monoamine synthesis and/or degradation within each region since we are exposing these animals during a dynamic developmental period.

Corticosterone. Only on P21 were there significant differences in plasma corticosterone; these differences were observed in animals that received MDMA, that is, the SAL+MDMA (mean ± SEM; 60.2 ± 7.3 ng/mL) and CIT+MDMA (43.4 ± 7.3 ng/mL) groups which showed significantly elevated corticosterone levels compared with the SAL+SAL group (15.2 ± 7.3 ng/mL). The CIT+SAL group (11.4 ± 7.3 ng/mL) did not differ from the SAL+SAL group (see Supporting Information Table S1). We previously showed increased corticosterone levels following developmental MDMA exposure when assayed on P12 and P16 after a single or multiple days of exposure to MDMA; it is also noteworthy that the 24 h

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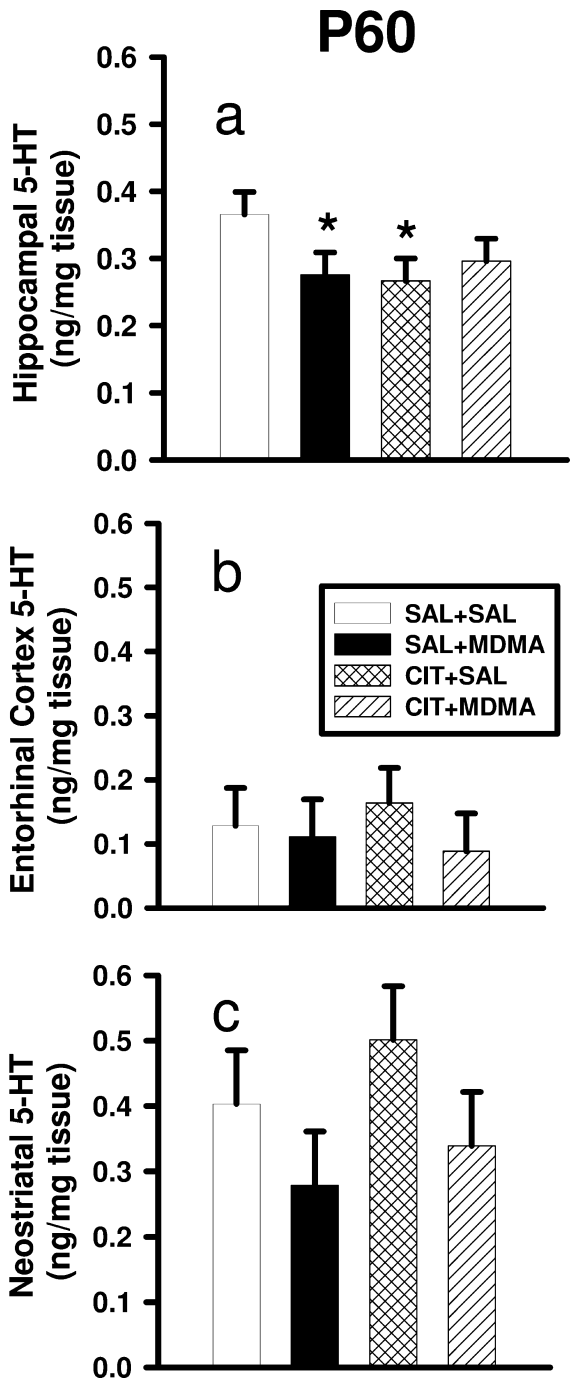

Figure 5. 5-HT levels at P60. Regional 5-HT levels in the (a) hippocampus, (b) entorhinal cortex (EC), and (c) neostriatum at P60 following P11−20 exposure. Both the SAL+MDMA and CIT+SAL groups had significantly decreased hippocampal 5-HT levels compared with the SAL+SAL group. No other effects were observed. (a) Main effect of treatment ($F(3,21) = 4.39$, $p < 0.02$), $n = 8$/treatment; (b) $n = 8$ for 2 × 5 CIT+SAL and $n = 6$ for other three treatment groups; (c) $n = 8$/treatment; *$p < 0.05$ different from SAL+SAL.

increase in corticosterone varies in terms of statistical significance as levels decline toward baseline at this time interval.[64,80] In sum, the data indicate that 2 × 5 CIT treatment does not have effects on corticosterone during development and would likely not be a confound in future behavior studies; however, a time-course analysis would be needed to ensure this at earlier time points. It is also possible that the current and previously reported corticosterone increases following MDMA administration play a role in altering long-term cognition; however, a study using neonatally adrenalectomized animals did not augment allocentric or egocentric learning in P11−20 methamphetamine exposed rat pups, suggesting an alternative mechanism of substituted amphetamine-induced deficits.[18]

NGF and BDNF. The only change we observed in hippocampal or neostriatal NGF or BDNF was seen in hippocampal NGF in the CIT+MDMA group on P16. The CIT+MDMA group had significantly higher NGF levels compared to the SAL+SAL and SAL+MDMA groups on P16 but not on P12 or P21. This increase may reflect increased stress but why it occurred at only one age is not clear. Hippocampal NGF reaches its peak between P12 and 21, and this may partially explain the effect only on P16. By contrast, neostriatal NGF was constant at all three ages, consistent with previous data.[19]

It was shown that BDNF levels in the hippocampus and neostriatum are increased following P11−20 administration of MDMA (2 × 20 mg/kg/day);[33] however, we did not show any changes in BDNF at any time-point in the current experiments. BDNF did not fluctuate over the 10 days analyzed, but it has been reported to increase during this interval by others.[14]

Overall, we replicated the previously published MDMA-induced 5-HT depletions (SAL+MDMA) in the hippocampus and neostriatum,[63,64,80] and this is the first study to show decreases in the EC. All regions showed a greater than 50% decrease in 5-HT after SAL+MDMA. Both the 2 × 5 and 1 × 10 CIT treatments attenuated MDMA-induced 5-HT reductions in the hippocampus on P21; however, two doses of 5 mg/kg CIT were used to provide partial protection to better match the MDMA exposure interval with the half-life of CIT.[48,51]

Others have reported significant depletions in SERT immunoreactivity in the hippocampus in both neonates and adult rats following P8−21 CIT exposure using the same daily dose of 10 mg/kg.[41,75] We also observed short and long-term serotonergic changes following CIT exposure (i.e., 2 × 5 CIT +SAL treatment reduced 5-HT levels in the hippocampus on P12 and on P60 (the latter after P11−20 exposure), as well as in the neostriatum and EC on P12 and P16, respectively). These changes during developmental exposure and in adulthood may indicate the potential of lasting learning and memory changes as a result of developmental CIT exposure alone. It is possible that both drugs could disrupt learning and memory due to increased 5-HT release, a common action of both drugs in adults. When looking at the severity of 5-HT depletions during dosing, there is only one instance when CIT produced a 5-HT depletion to the same degree as SAL +MDMA treatment (see Figure 4; neostriatum on P12) and the combination attenuated 5-HT depletions to levels significantly higher than SAL+MDMA treatment at all ages and in all brain regions with the exception of the EC on P21. Therefore, CIT shows promise as a method of partially interfering with the 5-HT reducing effect of neonatal MDMA exposure as the combination may be useful in testing the role of MDMA-induced early 5-HT reductions in the causal cascade that leads to later cognitive deficits.

METHODS

Animals and Housing. Nulliparous female Sprague−Dawley CD, International Genetic Strain, rats were obtained from Charles River Laboratories (Raleigh, NC) and mated with males from the same breeder. Pups from these matings were used as subjects. Rats were

housed in a 22 ± 1 °C environment at 50 ± 10% humidity with a 14/10 h light/dark cycle (lights on at 600 h). Prior to the animals being mated, a period of at least 1 week ensued to allow the animals to habituate to the conditions of the facility. Each polycarbonate cage (46 × 24 × 20 cm^3) contained wood chip bedding and ad libitum food and water, and was equipped with a stainless steel enclosure for environmental enrichment.[72] The Cincinnati Children's Research Foundation's Institutional Animal Care and Use Committee approved all protocols and the vivarium was fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). The day of birth was considered P0, and on P1 litters were culled to the appropriate number of males (8−10). The experiments described here were split-litter studies. Pups were individually identified by ear punch on P7 and weighed prior to every injection.

Only males were used for these studies for several reasons. First, we have previously shown only subtle, if any, drug × sex interactions on learning and memory following neonatal MDMA administration in rats.[7,66,67,73,74,79] Second, our model produces no significant differences between males and females in corticosterone or monoamine levels after neonatal MDMA exposure.[80] Third, we have observed that 2.5 mg/kg CIT treatment alone or in combination with MDMA on P11−20 does not produce sex differences in hippocampal 5-HT levels (unpublished observations). Lastly, we wanted to maintain similar litter sizes between the current study and previous experiments.

Drug Exposure. For all experiments, drugs were administered via subcutaneous injection with the location varied to avoid irritation to the dermis. *R,S*-Citalopram hydrobromide (CIT) (Sigma, St. Louis, MO) was used as a treatment, and 10 mg/kg (±)-3,4-methylenedioxyethamphetamine HCl (MDMA; National Institute on Drug Abuse) was administered every 2 h for a total of 4 injections on each day of treatment exposure in all experiments. CIT and MDMA were expressed as the freebase (purity >95%) and dissolved in isotonic SAL and administered in a volume of 3 mL/kg. SAL was used as the control vehicle for both CIT and MDMA.

Experiment 1 (Single CIT Treatment Injection, P11 MDMA Exposure Only, 24 h Assessment). For biochemical determinations, 9 litters consisting of 10 pups each were injected on P11 with a treatment of SAL or 1.25, 2.5, 5, or 10 mg/kg CIT 30 min prior to the first drug administration of MDMA or SAL. Males comprised the majority of all litters. MDMA or SAL was injected 4 times/day with a 2 h interdose interval; a total of 5 injections (1 CIT treatment plus 4 MDMA treatments, designating the CIT treatment first (SAL or CIT) and the MDMA treatment second (SAL or MDMA), the combinations resulted in the following groups (with balanced representation within each litter): (1) 1× SAL plus 4× SAL (SAL+SAL); (2) 1× SAL, plus 4 × 10 mg/kg MDMA (SAL+MDMA); (3) 1 × 1.25 mg/kg CIT plus 4× SAL (1.25 CIT+SAL); (4) 1 × 2.5 mg/kg CIT plus 4× SAL (2.5 CIT+SAL); (5) 1 × 5 mg/kg CIT plus 4 × 10 mg/kg SAL (5 CIT+SAL); (6) 1 × 10 mg/kg CIT plus 4 × 10 mg/kg SAL (10 CIT+SAL); (7) 1 × 1.25 mg/kg CIT plus 4 × 10 mg/kg MDMA (1.25 CIT+MDMA); (8) 2.5 mg/kg CIT plus 4 × 10 mg/kg MDMA (2.5 CIT+MDMA); (9) 5 mg/kg CIT plus 4 × 10 mg/kg MDMA (5 CIT+MDMA); (10) 10 mg/kg CIT plus 4 × 10 mg/kg MDMA (10 CIT+MDMA). At 24 h following the first MDMA dose on P11, animals were sacrificed (i.e., P12) and hippocampal 5-HT was assessed.

Experiment 2 (Single CIT Treatment Injection, P11−20 MDMA Exposure, 24 h Assessment). Based on the results from the previous experiment, the 2.5 mg/kg dose of CIT was chosen to determine its ability to prevent MDMA induced 5-HT depletions during the 10 day exposure period in which we have previously shown MDMA to cause long-term behavioral changes (P11−20). CIT was again given 0.5 h before the first of 4 doses of MDMA (given at 2 h intervals). Five litters were used (with group notation as above) and included the following treatment groups: (1) SAL+SAL; (2) SAL +MDMA; (3) 2.5 CIT+SAL; and (4) 2.5 CIT+MDMA. Animals were sacrificed on P21 (24 h following the first drug dose on P20). The hippocampus was dissected, frozen, and later analyzed for 5-HT and 5-HIAA content.

Experiment 3 (One or Two CIT Treatment Injections, P11−20 MDMA Exposure, 24 h Assessment). Seven litters were dosed from P11−20 with 1× or 2× treatments of 5 or 10 mg/kg CIT or SAL 30 min prior to the first and fourth treatments with MDMA or SAL. MDMA or SAL was injected 4 times/day with 2 h interdose intervals as before; a total of 6 injections per day (2 CIT treatments plus 4 MDMA or SAL treatments). Treatments represented within litter were as follows (using the same notation as above): (1) 2× SAL + 4× SAL (SAL+SAL); (2) 2× SAL + 4 × 10 mg/kg MDMA (SAL+MDMA); (3) 1 × 5 mg/kg CIT + 4× SAL (1 × 5 CIT+SAL); (4) 1 × 10 mg/kg CIT + 4× SAL (1 × 10 CIT+SAL); (5) 1 × 5 mg/kg CIT + 4 × 10 mg/kg MDMA (1 × 5 CIT+MDMA); (6) 1 × 10 mg/kg CIT + 4 × 10 mg/kg MDMA (1 × 10 CIT+MDMA); (7) 2 × 5 mg/kg CIT + 4× SAL (2 × 5 CIT+SAL); (8) 2 × 10 mg/kg CIT + 4× SAL (2 × 10 CIT+SAL); (9) 2 × 5 mg/kg CIT + 4 × 10 mg/kg MDMA (2 × 5 CIT+MDMA); (10) 2 × 10 mg/kg CIT + 4 × 10 mg/kg MDMA (2 × 10 CIT+MDMA). Animals that received the 1× CIT treatments were injected with SAL for the second treatment dose (0.5 h prior to the last dose of MDMA or SAL) to maintain a total of 6 injections/day for all animals. On P21, animals were sacrificed for monoamine assessment. All animals were assayed for hippocampal 5-HT, and only those that received SAL+SAL, 2 × 5 CIT+SAL, SAL+MDMA, or 2 × 5 CIT+MDMA were assayed for hippocampal 5-HIAA and utilization ratios, neostriatal 5-HT, 5-HIAA, DA, DOPAC, and utilization ratios, EC 5-HT, 5-HIAA, and utilization ratios, hippocampal and neostriatal NGF and BDNF, and corticosterone in plasma.

Experiment 3 (Two CIT Treatments, P11 Only or P11−15 MDMA Exposure, 24 h Assessment). It was determined that the optimal treatment dosing regimen for attenuating the reductions in hippocampal 5-HT after 10 days of MDMA administration was 2 × 5 CIT. Therefore, an additional 8 litters were treated using the same daily 6 injection dosing regimen with 2 pups from each litter receiving SAL+SAL, SAL+MDMA, 2 × 5 CIT+SAL, or 2 × 5 CIT+MDMA. One pup from each treatment was treated on P11 only and assayed on P12 and the remaining 4 pups were dosed from P11−15 and assayed on P16. These exposure periods (P11 and P11−15) were chosen to ensure that the treatment doses of CIT attenuated MDMA induced 5-HT reductions at the beginning and halfway through the 10-day exposure period known to produce behavioral deficits. The 24 h assay time was used following each treatment regimen because we have previously shown the most dramatic decrease in hippocampal 5-HT following P11 MDMA exposure at this time point with continued decreases on P16.[63,64,80]

The same brain regions, monoamines, growth factors, and hormones assayed for the SAL+SAL, SAL+MDMA, 2 × 5 CIT +SAL, and the 2 × 5 CIT+MDMA groups that received P11−20 exposure were assayed following P11 only or P11−15 exposure.

Experiment 3 (Two CIT Treatment Injections, P11−20 MDMA Exposure, Adult Assessment). After verification that a treatment of 2 × 5 CIT+MDMA attenuated MDMA-induced 5-HT depletions in the hippocampus, neostriatum, and EC on P12, P16, and P21, we assessed 5-HT, DA, and metabolites in the same brain regions in adult animals (P60) following P11−20 exposure. Eight litters were used for this experiment, and the four treatment groups were as above.

Blood and Tissue Collection. At the designated time point, each animal was removed from the home cage and within 30 s was decapitated and blood was collected in tubes containing 2% EDTA (0.05 mL) and centrifuged (1399 RCF) for 25 min at 4 °C. Plasma was collected and stored at −80 °C until assayed for corticosterone.

The brain was simultaneously removed and placed in a chilled brain block (Zivic-Miller, Pittsburgh, PA) to aid in dissection of the neostriatum and hippocampus as described previously.[78] For the neostriatum, a coronal cut was made at the optic chiasm and another 2 mm rostral to the first. The neostriatum (caudate-putamen) was dissected from this 2 mm section. The EC was dissected by making a coronal cut at the posterior extent of the mammillary body and another 2 mm posterior to the first. From this 2 mm section, the EC was removed bilaterally by making a cut at the rhinal fissure and removing the cortical tissue inferior to this cut to the tip of the corpus callosum. Hippocampi were removed from the remaining tissue.

Tissues were immediately frozen on dry ice and stored at −80 °C until monoamines were quantified by high-pressure liquid chromatography with electrochemical detection.

Monoamine Determinations. For neonatal samples, the tissue concentrations of DA, DOPAC, 5-HT, and 5-HIAA in the neostriatum and 5-HT and 5-HIAA in the hippocampus and EC were quantified using high-pressure liquid chromatography with electrochemical detection.[63] Tissue weights were determined prior to homogenization in 50 volumes of 0.2 N perchloric acid and centrifuged for 6 min at 10,000*g* RCF. Aliquots of 20 μL were injected onto a C18-column (MD-150, 3 × 150 mm; ESA, Chelmsford, MA) connected to a Coulochem detector (25A, Chemsford, MA), and an integrator recorded the peak heights for each injection. The potentials of the E1 and E2 on the analytical cell (model 5014B) of the Coulochem were −150 and 160 mV, respectively. The mobile phase consisted of 35 mM citric acid, 54 mM sodium acetate, 50 mg/L disodium ethylenediamine tetraacetate, 70 mg/L octanesulfonic acid sodium salt, 6% (v/v) methanol, and 6% (v/v) acetonitrile, pH 4.0, and was pumped at a flow rate of 0.4 mL/min. Quantification of analytes was calculated on the basis of standards. Retention times for DOPAC, DA, 5-HIAA, and 5-HT were approximately 6, 8, 11, and 17 min, respectively. For adult samples, a slightly modified version was used.[20]

Corticosterone Assessment in Plasma. Plasma was diluted 3:1 in supplied assay buffer, and corticosterone levels (ng/mL) were assayed in duplicate using a commercially available EIA (Immunodiagnostic Systems Inc., Fountain Hills, AZ) that was read on a SpectraMax Plus (Molecular Devices, Sunnyvale, CA). The corticosterone EIA has little cross-reactivity with other hormones or precursors (<1.4%) with the minor exceptions of 11-dehydrocorticosterone and 11-deoxycorticosterone (<6.7%).

NGF and BDNF Assessment. The concentrations of NGF and BDNF in the hippocampus and neostriatum were determined on P12, P16, and P21 using the Emax ImmunoAssay System (Promega Corp, Madison, WI). The samples were homogenized in lysis buffer (1 mL) according to kit instructions, and hippocampal samples were further diluted 1:2 and the neostriatal samples 1:10 prior to assay. All samples were assayed in duplicate according to the manufacturer's instructions, and levels were expressed against total protein (i.e., pg/mg protein). Protein was assayed using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Optical densities were measured on a SpectraMax Plus microtiter plate reader (Molecular Devices, Sunnyvale, CA).

Statistical Analyses. Monoamines, corticosterone, BDNF, and NGF were analyzed using ANOVA, and then posthoc analysis was performed using Dunnett's *t* test after each dosing regimen, and significance was reported for treatments that were different from SAL +SAL or SAL+MDMA treatments. Significance was set at $p \leq 0.05$. Data are presented as least-squares (LS) means ± LS SEM.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table S1: 5-HIAA, 5-HIAA/5-HT ratio, DA, DOPAC, DOPAC/DA ratio, NGF, BDNF, and corticosterone on P12, 16, and 21. Table S2: 5-HIAA, 5-HIAA/5-HT ratio, DA, DOPAC, DOPAC/DA ratio, on P60. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

T.L.S. assisted in experimental design, dosed the rats, collected tissue, ran HPLC, analyzed data, and wrote the manuscript. C.E.G., M.R.S., D.L.G. dosed the rats and collected tissue. GAG was responsible for HPLC analysis. C.V.V. and M.T.W. assisted in experimental design, analysis, and manuscript preparation.

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Notes

Abbreviations:

5-HT, serotonin; MWM, Morris water maze; CWM, Cincinnati water maze; MDMA, ecstasy; CIT, citalopram; EC, entorhinal cortex

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