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Neuronal reorganization in adult rats neonatally exposed to (±)-3,4-methylenedioxymethamphetamine

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ABSTRACT

The abuse of methylenedioxymethamphetamine (MDMA) during pregnancy is of concern. MDMA treatment of rats during a period of brain growth analogous to late human gestation leads to neurochemical and behavioral changes. MDMA from postnatal day (P)11–20 in rats produces reductions in serotonin and deficits in spatial and route-based navigation. In this experiment we examined the impact of MDMA from P11 to P20 (20 mg/kg twice daily, 8 h apart) on neuronal architecture. Golgi impregnated sections showed significant changes. In the nucleus accumbens, the dendrites were shorter with fewer spines, whereas in the dentate gyrus the dendritic length was decreased but with more spines, and for the entorhinal cortex, reductions in basilar and apical dendritic lengths in MDMA animals compared with saline animals were seen. The data show that neuronal cytoarchitectural changes are long-lasting following developmental MDMA exposure and are in regions consistent with the learning and memory deficits observed in such animals.

1. Introduction

(±)-3,4-Methylenedioxymethamphetamine (MDMA) can alter neurotransmitter and hormonal systems [1] as well as impair cognitive function among users. These cognitive changes in users can occur not only during the period of drug ingestion but for protracted periods thereafter [2–7]. Worldwide, the largest demographic to abuse MDMA is young adults of reproductive age [8,9]. Those who take MDMA may be at risk for unintended pregnancy [10–14]. In consideration of the negative effects produced by MDMA in adults, there is concern that fetal exposure to MDMA may adversely affect children's physical and/or cognitive development. Nonetheless, few human studies have been undertaken to examine the impact of MDMA during gestation on neurodevelopmental outcomes, even though such exposures are known to occur [12,15–17], as MDMA easily crosses the placenta [18]. Most studies have examined children only within the perinatal period for physical abnormalities and not for cognitive function [16,17]. A newer study examined early mental development following prenatal MDMA exposure and found the amount of drug exposure predicted poorer outcomes when the children were assessed within their first year of life [19]. Another substituted amphetamine

Abbreviations: 5-HT, serotonin; ANOVA, analysis of variance; CWM, Cincinnati water maze; DG, dentate gyrus; EC, entorhinal cortex; MDMA, methylenedioxymethamphetamine; MWM, Morris water maze; NAcc, nucleus accumbens; P, postnatal day; SAL, saline.

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of abuse, methamphetamine, has been shown to result in deficits in cognitive function, motoric performance at 1 year, and volumetric and physiological changes to regions of the brain in children following gestational exposure [20–24]. As MDMA demonstrates different effects on neurotransmitter and hormonal systems when compared with methamphetamine, it is unknown whether similar changes occur in children exposed in utero to MDMA.

In rats it has been shown that MDMA exposure, during a period of brain development similar to late gestational human in utero brain development [25], produces cognitive deficits in the Morris water maze (MWM), a test of spatial learning and memory and the Cincinnati water maze (CWM), a test of egocentric route-based learning [26–32]. These effects are not the result of MDMA-induced body weight reductions, injection stress, or changes in maternal care [32]. Furthermore, the deficits emerge early and for spatial learning are evident for at least a year [28]. Interestingly, some of these effects may be dependent upon the pattern of drug administration [31]. The underlying mechanisms for these changes, however, are not known. There is evidence that this exposure (postnatal days (P)11–20) causes reductions in monoamines during both the neonatal period and in adulthood [26,33–36]. Further, neonatally MDMA-exposed animals have reductions in cAMP-dependent protein kinase A activity and altered 5-HT_{1A} receptor activation when assessed in adulthood [33]. It is not known whether these biochemical effects are accompanied by neuroanatomical changes in regions important for spatial learning or egocentric learning.

Spatial learning in the MWM requires a properly functioning hippocampus [37] and related regions that participate in allocentric mapping (e.g., [38]). Route-based learning, on the other hand, requires primarily non-hippocampal regions, including the entorhinal cortex (EC), dorsal striatum, and presubiculum [39–41]. The purpose of this experiment was to investigate whether long-term changes in the cytoarchitecture of brain regions important in learning, memory, and reinforcement occur after a dosing regimen of MDMA that is known to induce learning and memory deficits. The nucleus accumbens (NAcc) was included since we and others showed this to be a vulnerable area to amphetamines [42–44], and data showing that the NAcc can affect both spatial and egocentric learning and memory [45–49]. Doses were selected to be comparable to human prenatal exposure based on an interspecies exposure scaling algorithm (reviewed in [29]). Because we have shown that a number of control procedures such as injection stress and body weight reductions do not play a role in the long-lasting learning and memory deficits following MDMA [32], we limited comparisons to MDMA and the vehicle-treated saline controls and did not include uninjected controls.

2. Material and methods

2.1. Subjects

Prior to experimentation, all procedures were approved by the Cincinnati Children's Research Foundation's Institutional Animal Care and Use Committee and all efforts were taken to minimize discomfort and/or pain and conformed to the National Institutes of Health guidelines for animal use. The vivarium was fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The vivarium was maintained on a 14 h light/10 h dark cycle (lights on at 600 h) and food and water were freely available. Nulliparous female and male Sprague–Dawley CD International Genetic Standard rats (Charles River Laboratories, Raleigh, NC) were mated in hanging wire cages following an acclimation period to the vivarium of at least 1 week. The day a sperm plug was detected was considered embryonic day 0. Fourteen days after being placed with the males, females were transferred to polycarbonate cages (46 cm × 24 cm × 20 cm) with woodchip bedding. The day of birth was considered P0 and on P1 litters were standardized to 8 pups balanced for 4 males and 4 females to the extent possible. Offspring were weighed weekly and uniquely identified by an ear punch on P11. The offspring were the subjects for these experiments and were randomly selected for inclusion using a random numbers table. Lab personal were blind to treatment group assignment.

2.2. (±)-3,4-Methylenedioxymethamphetamine treatment

(±)-3,4-Methylenedioxymethamphetamine HCl (expressed as the freebase and greater than 95% pure) was obtained from the National Institute on Drug Abuse through its provider Research Triangle Institute (Research Triangle Park, NC). MDMA was dissolved in sterile isotonic saline (SAL) at a concentration of 20 mg/3 ml and administered to animals at a dose of 20 mg/kg through a subcutaneous route in the dorsum. MDMA was delivered twice daily from P11 to P20 with an 8 h interval between treatments. Irritation to the dermis was minimized by varying injection sites. Prior to each injection, animals were weighed. Animals were weaned naturally by their dams [50,51] and separated on P28 and housed in pairs. A total of six litters were prepared for this study and only a single pair of males (1 MDMA-treated and 1 SAL-treated) was used from each litter in a split-litter design.

2.3. Golgi–Cox analysis

On approximately P60 (a day that is encompassed by our previous behavioral testing periods, see [29]) the animals were given an overdose of sodium pentobarbital and perfused intracardially with physiological saline using a Masterflex pump (Cole Palmer, Vernon Hills, IL). Littermates (i.e., 1 MDMA and 1 SAL animal) were perfused simultaneously and following 10 min of perfusion, whole brains were removed. Brains were placed in Golgi–Cox solution for 14 days and subsequently in a Golgi–Cox/30% sucrose solution for 3 days. The tissues were cut in 200 μm sections using a vibratome, and sections were mounted on gelatinized slides and stained using the method described previously [52]. Neurons were identified at a lower magnification (100×) in each hemisphere and then five neurons per hemisphere in each region of interest were traced at a higher magnification (250×) using the camera lucida method. An experimenter, blinded to treatment, performed

the cell selection and tracings. In order for a neuron to be selected it had to have a dendritic tree that was well visualized and not obscured by stain precipitate, blood vessels, or astrocytes. If more than five neurons were identified in each hemisphere as reaching these criteria, then the cells included were randomly selected from the total number of cells identified. Four brain areas were traced and quantified, these were as follows: EC (Layer II), nucleus accumbens shell (NAcc), and the dentate gyrus (DG), and only dendritic lengths were examined in Layer V of the medial frontal cortex (Zilles Cg3). Pyramidal cells were traced in the cortical regions, granule cells in the DG, and medium spiny neurons in the NAcc. These regions were selected based on the behavioral phenotype that animals display following exposure to MDMA during the same neonatal period as used here, namely impairments in spatial learning and route-based egocentric learning [26–28,31,32].

Dendritic length was quantified using a Sholl analysis of ring intersections [53]. For example, to obtain a measure of length and complexity, the total number of ring intersections was summed and then averaged across cells. Spine density was quantified by counting spines on one fourth-order terminal tip from basilar dendrites and one third-order terminal tip from apical dendrites in the cortex. These regions of the dendrites were used for analysis since this is typically where differences have been observed in past studies following psychostimulant administration in adult and developing rats [43,44,54]. Secondly, when there are increases in dendritic length it most often occurs in the distal end, thus, increasing the likelihood of changes in dendritic spine densities at this location. Neither NAcc nor DG neurons have apical and basilar dendritic trees, therefore, spine density was quantified using a third-order terminal tip in these regions. Spine density was consistently performed on the same branch order and counted as the number of spines/10 μm of the dendritic branch. The magnification factor for the analysis of spine density was 1000×. The morphological analyses were performed by personnel blind to the treatment group of the animals.

2.4. Statistics

Because litter effects are an important source of variance in developmental studies [55], data were analyzed using a mixed general linear model analysis of variance (ANOVA; SAS 9.1 Proc Mixed, Cary, NC). In this model, litter was a blocking factor. For some brain regions, neurons were either not stained completely or no staining was present and therefore the number of animals represented was 4–6/treatment. With litter accounted for in the block factor, treatment was a between factor and hemisphere was a repeated measure factor. Significant interactions were analyzed using *t*-tests generated by Proc Mixed. Proc Mixed provides adjusted degrees of freedom using the Kenward–Roger method. Specific regions were analyzed separately as were basilar and apical dendritic trees. Data are presented as mean ± SEM and statistical significance was accepted at $p \leq 0.05$ and only significant Treatment or Treatment interactions are reported.

3. Results

Fig. 1 shows camera lucida drawings of representative neurons in the NAcc (Fig. 1A and B), DG (Fig. 1C and D), and EC (Fig. 1E and F) for both SAL- and MDMA-treated animals.

In the NAcc, there were significant effects of Treatment ($F(1,5) = 49.55$, $p < 0.001$). The MDMA-treated animals had shorter dendritic lengths than the SAL-treated animals (Fig. 2 top). Similarly, the MDMA-treated animals had fewer dendritic spines than the SAL-treated animals ($F(1,5) = 9.06$, $p < 0.03$ (Fig. 2 bottom)). No interaction for dendritic length or number of spines was observed.

In the DG the dendritic length was shorter in the MDMA-treated animals [Treatment, $F(1,10.2) = 5.89$, $p < 0.04$ (Fig. 3 top)], whereas the number of spines was increased in the MDMA-treated animals compared with SAL-treated animals [Treatment, $F(1,4.2) = 9.97$, $p < 0.04$ (Fig. 3 bottom)]. There was a significant interaction of Treatment × Hemisphere ($F(1,9) = 9.75$, $p < 0.02$), however, irrespective of laterality, both hemispheres in the MDMA-treated animals showed an increase in spine number compared with the SAL-treated animals (not shown).

In the EC there were significant effects of Treatment for both the basilar ($F(1,5) = 18.1$, $p < 0.01$) and apical ($F(1,5) = 13.97$, $p < 0.02$) dendritic lengths. The MDMA-treated animals had a reduction in dendritic length in both the basilar (Fig. 4A) and apical (Fig. 4B) branches compared with SAL-treated animals. No other differences were observed for either the basilar or apical dendrites. Contrary to the differences in length, no differences were observed for the number of basilar (Fig. 4C) or apical (Fig. 4D) spines.

In order to determine if the changes in dendritic length were specific to the EC we also examined the length of the basilar and apical branches in the frontal cortex. Unlike the EC, no differences in dendritic length were observed in the frontal cortex between MDMA- and SAL-treated animals (not shown).

4. Discussion

Exposure to MDMA in neonatal rats leads to persistent deficits in the ability of the animals to perform similarly to control animals in various cognitive tests. Specifically, deficits are observed in spatial learning in the MWM and Barnes maze, in the CWM when run under bright light with visible distal cues and when run under infrared light with no visible cues (see [29]). Taken together, the data suggest that multiple regions of the brain are permanently affected by MDMA administration during the neonatal period. Because of the known learning deficits, we examined several regions of interest, including the DG, NAcc, and EC, and show that long-term changes in neuronal architecture are present in these regions, regions important for spatial and egocentric learning and memory, and at an age when learning and memory deficits have previously been demonstrated.

While mechanisms underlying the deficits in learning and memory are unknown following neonatal MDMA exposure, there are disruptions to multiple systems during and after the exposure period used here. For example, we have demonstrated that monoamines and especially

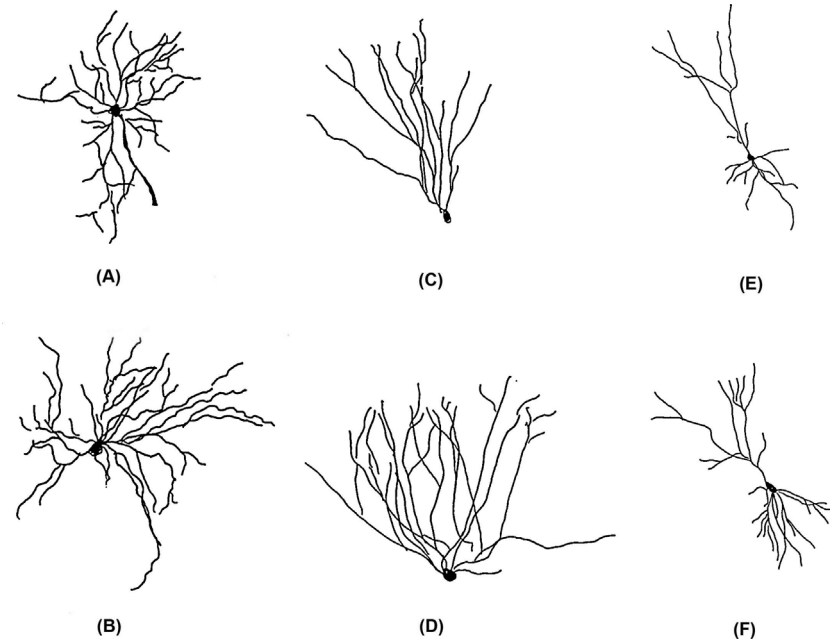

Fig. 1. Displayed are representative camera lucida tracings of nucleus accumbens pyramidal cells (A and B), dentate gyrus granular cells (C and D), and neurons from Layer II of the entorhinal cortex (E and F) from animals treated neonatally with MDMA (A, C, and E) or SAL (B, D, and F).

serotonin (5-HT) are depleted both during and following neonatal exposure to MDMA [33–36]. This reduction appears to fluctuate over time but remains decreased in the hippocampus through adulthood. Others have demonstrated that exposure on P3 or P4 to agents that cause transient (para-chloroamphetamine) or permanent (5,7-dihydroxytryptamine) depletions in 5-HT produces reductions in spines without changes to the length of the neuron in the DG [56]. In contrast, in the present experiment MDMA administration increased the number of spines in this region and decreased length. While the timing of the 5-HT depletions differs between these studies by almost a week, there may be factors other than 5-HT that are affecting the MDMA-treated animals. MDMA is not a purely serotonergic drug and can affect other neurotransmitter systems (Rothman et al., 2001). While dopamine levels are similar to controls during the neonatal period of MDMA administration, 3,4-dihydroxyphenylacetic acid levels are changed [36,57], suggesting an impact of MDMA on dopamine metabolism. In adulthood, the animals treated as neonates with MDMA show decreases in dopamine in the prefrontal cortex and striatum [33], but see [27]. This change in dopamine may be important since we have shown that drugs that deplete dopamine in the striatum in adulthood produce learning and memory deficits in the CWM [58,59]. Therefore, either the early changes in dopaminergic metabolism, or the later decreases in dopamine, may underlie learning deficits in the CWM. It should be noted that animals treated with MDMA from P11 to P20 also demonstrate hypoactivity [27,60], further implicating dopamine as playing some role in cognitive deficits produced by neonatal MDMA despite the fact that locomotor hypoactivity did not translate to any changes in swim speed during water maze testing.

We have shown that neonatal rats treated with methamphetamine from P11 to P20 have similar changes in the NAcc as those treated with MDMA during this same period; that is, decreased dendritic length and spine density. MA-treated and MDMA-treated animals both had an ~16% decrease in dendritic length in the NAcc, whereas for number of spines, MA-treated animals had an ~10% decrease and the MDMA-treated animals had an ~17% decrease. In the DG, the two drugs produced different effects. For example, MA only produced an ~11% decrease in spines in the DG, whereas MDMA produced an ~45% increase in spine density in this region and an ~13% decrease in length. Interestingly, MDMA-treated animals tend to have greater deficits than MA-treated animals in the MWM (see [35,44] for comparison), a hippocampally modulated behavior [37,61]. It should also be pointed out that when there were differences in the magnitude of an effect, the MDMA-treated animals appear to be more affected than MA-treated animals. Animals that were exposed to a developmental stressor, maternal separation from P3 to P21, show increased spine density in the NAcc [62], lending further support to the idea that the changes observed here are biologically relevant. Neither drug had an effect on the frontal cortex. The EC was not examined following methamphetamine exposure so a comparison between the two drugs cannot be made for this region. The EC is a region important in egocentric learning and memory [41,63]. The changes in the EC may be important for explaining the learning deficits in the CWM. This is the first study to our knowledge to examine the EC of adult rats that were neonatally exposed to a substituted amphetamine.

In adult animals, others have examined the change in neuronal architecture following multiple days of MDMA exposure. Whereas dendritic length in the NAcc was not

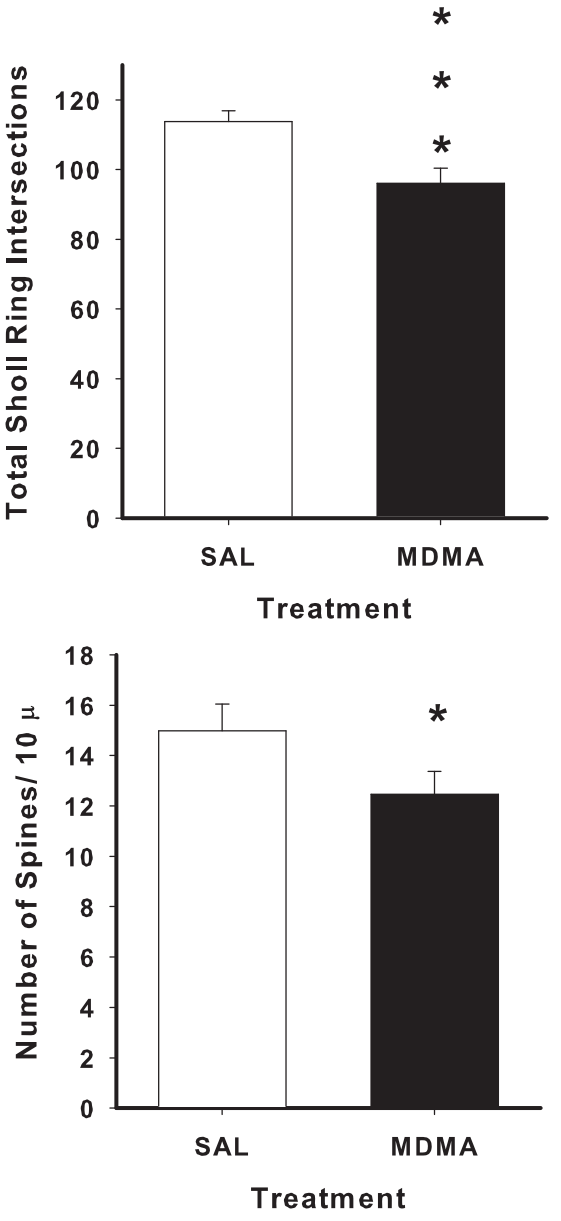

Fig. 2. Total Sholl ring intersections in the NAcc (top) were decreased in animals treated with MDMA from P11 to P20 compared with saline (SAL) animals ($p < 0.001$), and the number of spines (bottom) in this region was also decreased in the MDMA-treated animals versus SAL animals ($p < 0.05$). $^{*}p < 0.05$, $^{***}p < 0.001$.

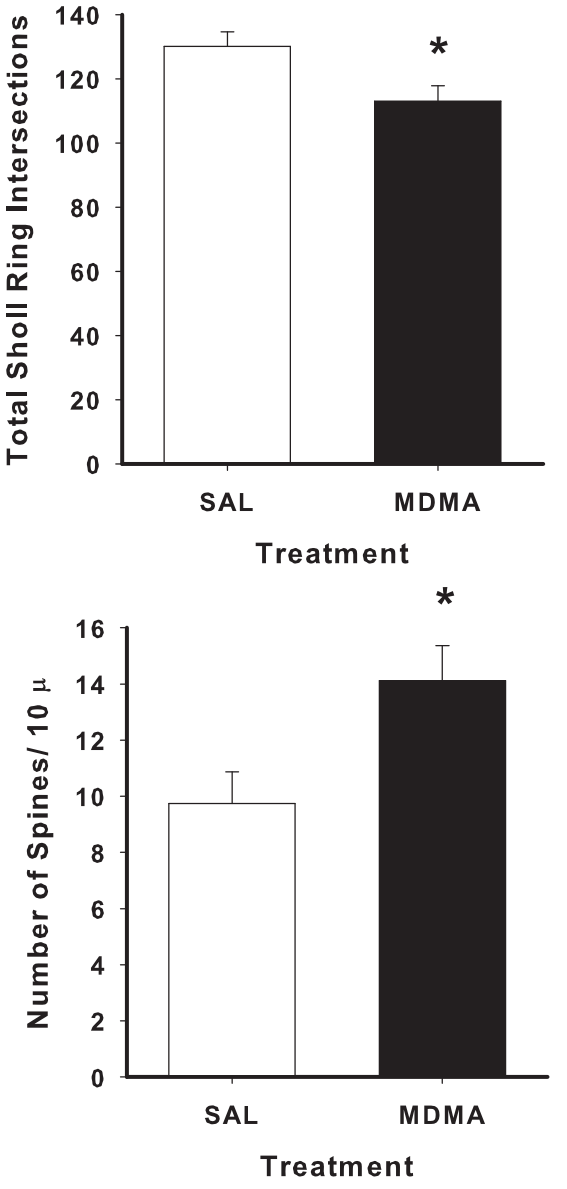

Fig. 3. Total Sholl ring intersections in the dentate gyrus (top) were decreased in animals treated with MDMA from P11 to P20 compared with saline (SAL) controls ($p < 0.05$), whereas the number of spines (bottom) in this region was increased in the MDMA-treated animals versus SAL control ($p < 0.05$). $^{*}p < 0.05$.

changed in adult MDMA-treated animals, the number of spines in the shell was increased [64]; a similar effect was demonstrated in the dorsal striatum of adult animals that were administered MDMA [65]. Furthermore, adult animals have small changes in the prefrontal cortex for the composition of neurons and the number of spines, where the effect (i.e., increases or decreases) in the number of spines was dependent upon the branching of the dendrite [64]. Adult animals treated with MDMA do not demonstrate egocentric route-based learning and memory deficits [59] or spatial learning deficits [66] as seen in animals treated neonatally (see [29]). Rather, adult animals have deficits in the CWM only when tested with distal cues present but not when distal cues are removed [66,67]. While procedural differences may account for the differences in effects between adult and neonatal administration of MDMA, the most important difference appears to be the period of development when animals are treated. Taken together, these data implicate the decreases in spine number and dendritic length as factors that may contribute to the learning and memory deficits of animals treated neonatally with MDMA. The changes in morphology of Golgi-stained dendrites provide

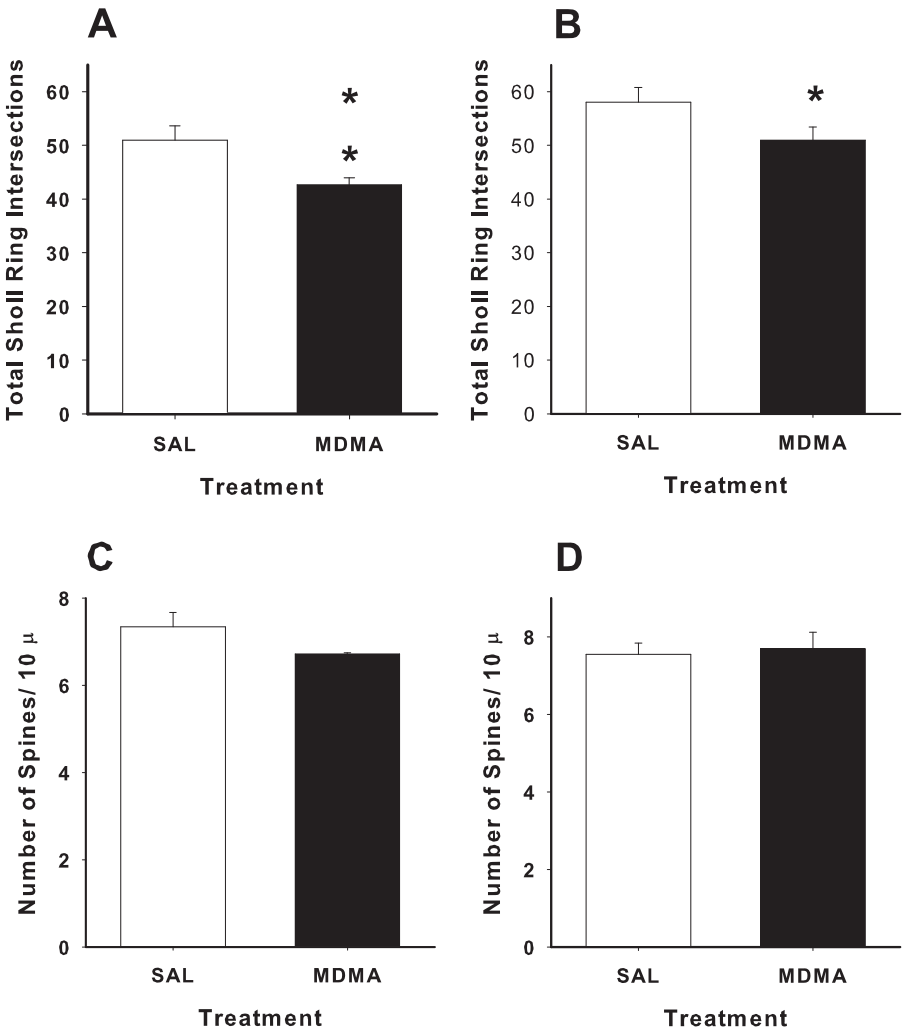

Fig. 4. In the entorhinal cortex total Sholl ring intersections were decreased in both the basilar (A) and apical (B) dendrites in neonatally MDMA-treated animals compared with SAL-treated animals ($p < 0.01$ and $p < 0.05$, respectively). No differences were noted in the number of spines in either the basilar (C) or apical (D) projections. $^{*}p < 0.05$, $^{**}p < 0.01$.

initial evidence of altered synaptic connectivity [42]. In support of the idea that changes in dendritic spines are affected by learning, in humans, the amount of education a person has correlates with dendritic length, such that those with more than a high school education have longer dendritic lengths than those with only a high school education, and these both have longer dendritic lengths than those without a high school education [68]. In addition, it has been suggested that changes in dendritic synaptic morphology are indicative of a particular disease state for various neurological disorders whether, for example, from Alzheimer's disease or drug addiction [69]. This is relevant to the present study since neonatal MDMA exposure produces allocentric and egocentric learning impairments concurrently with decreases in dendritic length and spine density in brain regions implicated as important for these two types of learning and memory. The hippocampus, prefrontal cortex, and entorhinal cortex all project to the NAcc (for review, [70]), and the changes observed in the NAcc may point to synaptic connectivity changes that impair learning and memory in MDMA-treated animals.

There are limitations to this study. The animals were examined in adulthood, therefore the developmental time course for these cytoarchitectural changes is unknown. However, if the morphological changes precede the learning and memory deficits, then learning and memory changes should appear very early and indeed we have shown that allocentric and egocentric learning and memory deficits in MDMA-treated animals are present as early as P30 [28]. We used a single dose of MDMA and only one exposure period. We used this dose because it consistently produces learning and memory deficits when given as a single or divided daily dose during the P11–20 exposure period [31]. We did not examine animals after learning and memory testing to determine if the morphological changes could be directly correlated to the learning and memory deficits; this must await further study now that morphological effects have been found.

In conclusion, the results show that neonatal MDMA exposure produces specific changes within the brain in regions that play essential roles in allocentric and egocentric learning and memory. It is recognized that Golgi staining is suggestive for loss or increase of synaptic connectivity, and that further investigation is needed. Nonetheless, the changes in spine densities and dendritic lengths suggest long-term synaptic reorganization following early MDMA exposure during a period that is approximately analogous to late gestational human brain development.

Transparency document

The Transparency document associated with this article can be found in the online version.

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