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Prenatal L-DOPA exposure produces lasting changes in brain dopamine content, cocaine-induced dopamine release and cocaine conditioned place preference

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ABSTRACT

Dopamine, its receptors and transporter are present in the brain beginning from early in the embryonic period. Dopamine receptor activation can influence developmental events including neurogenesis, neuronal migration and differentiation raising the possibility that dopamine imbalance in the fetal brain can alter development of the brain and behavior. We examined whether elevated dopamine levels during gestation can produce persisting changes in brain dopamine content and dopamine-mediated behaviors. We administered L-3,4-dihydroxyphenylalanine (L-DOPA) in drinking water to timed-pregnant CD1 mice from the 11th day of gestation until the day of parturition. The prenatal L-DOPA exposure led to significantly lower cocaine conditioned place preference, a behavioral test of reward, at postnatal day 60 (P60). However, in vivo microdialysis measurements showed significant increases in cocaine-induced dopamine release in the caudate putamen of P26 and P60 mice exposed to L-DOPA prenatally, ruling out attenuated dopamine release in the caudate putamen as a contributor to decreased conditioned place preference. Although dopamine release was induced in the nucleus accumbens of prenatally L-DOPA exposed mice at P60 by cocaine, the dopamine release in the nucleus accumbens was not significantly different between the L-DOPA and control groups. However, basal dopamine release was significantly higher in the prenatally L-DOPA exposed mice at P60 suggesting that the L-DOPA exposed mice may require a higher dose of cocaine for induction of cocaine place preference than the controls. The prenatal L-DOPA exposure did not alter cocaine-induced locomotor response, suggesting dissociation between the effects of prenatal L-DOPA exposure on conditioned place preference and locomotor activity. Tissue concentration of dopamine and its metabolites in the striatum and ventral midbrain were significantly affected by the L-DOPA exposure as well as by developmental changes over the P14–P60 period. Thus, elevation of dopamine levels during gestation can produce persisting changes in brain dopamine content, cocaine-induced dopamine release and cocaine conditioned place preference.

1. Introduction

Dopamine plays critical roles in the regulation of motor and cognitive functions in the mature CNS. However, dopamine and its receptors first appear in the brain early in the embryonic period before mature synaptic contacts form (Araki et al., 2007; Ohtani et al., 2003) and modulate developmental processes such as neurogenesis, neuronal migration and differentiation (Bhide, 2009; Crandall et al., 2007; Frederick and Stanwood, 2009; Levitt et al., 1997; Nguyen, 2001; Ohtani et al., 2003; Popolo et al., 2004; Stanwood et al., 2006). Since dopamine can influence developmental processes, it is reasonable to assume that environmental or genetic factors that perturb dopamine receptor signaling in the developing CNS can perturb normal brain development. Indeed, neurological and neuropsychiatric disorders of developmental origin such as attention deficit hyperactivity, schizophrenia and autism spectrum disorder are associated with impairment of dopaminergic neurotransmission (Biederman, 2005; Bloom, 1993; Weinberger, 1995; Benes, 2000; Schwartz et al., 2000).

A general finding in these developmental disorders is the lack of readily discernible structural changes in the brain such as loss or

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mal-positioning of neurons, despite significant alteration of neuro-cognitive function. This finding is in sharp contrast to that in neurodegenerative diseases or neuronal migration disorders, in which neuronal loss or mal-positioning is a characteristic feature, along with neuro-cognitive impairment. Therefore, dopamine imbalance during brain development may lead to disorders of neurological function rather than structure. In support of this view, we found that elevated dopamine content in the embryonic brain produced by administration of L-DOPA to pregnant dams did not produce significant changes in the size of dopamine-rich regions of the brain such as the caudate putamen or frontal cortex or alter total numbers of neurons or glial cells in these brain regions (McCarthy et al., 2007). However, the L-DOPA exposure produced significant changes in neurogenesis in the lateral ganglionic eminence (precursor of cells of the caudate putamen and nucleus accumbens) and frontal cortical neuroepithelium in the embryonic period (McCarthy et al., 2007; Ohtani et al., 2003) leading to subtle changes in neuronal numbers in the caudate putamen, nucleus accumbens and frontal cortex that could be revealed only by neuronal birth-dating methods (McCarthy et al., 2007).

Whether the subtle cellular changes revealed by the neuronal birth-dating methods were sufficient to produce functional changes remained uncertain. In the present study, we show that adult offspring born to mothers exposed to L-DOPA during pregnancy show significant changes in striatal dopamine content, cocaine-induced dopamine release and cocaine conditioned place preference. Therefore, we suggest that prenatal L-DOPA exposure produces lasting changes in neurological function without producing readily discernible structural changes in the brain and suggest that the prenatal L-DOPA exposure mouse model may be instructive for studying the pathophysiology of neuro-functional disorders associated with developmental dopamine imbalance.

2. Experimental procedures

2.1. Animals

We used timed-pregnant CD1 mice (Charles River Laboratories, Wilmington, MA) housed in our institutional animal facility. The day of vaginal plug discovery was designated embryonic day 0 (E0) and the day of birth postnatal day 0 (P0). All experimental procedures were in full compliance with institutional and NIH guidelines for the care and use of laboratory animals.

2.2. Administration of L-DOPA to timed-pregnant mice

The dopamine precursor L-DOPA (Sigma Chemical Co. St. Louis. MO) was administered to pregnant mice in drinking water from the 11th day of pregnancy until parturition. L-DOPA (2 mg/ml) was dissolved in drinking water containing 0.025% ascorbic acid (Sigma). Ascorbic acid prevents oxidation of L-DOPA in the water (Marien et al., 1984; Porter et al., 1999; Schmidt et al., 1996). Control groups received drinking water containing 0.025% ascorbic acid alone or drinking water without additives. Thus, we created 3 groups of mice: mice that received L-DOPA plus ascorbic acid (L-DOPA), mice that received ascorbic acid alone (ASC) and mice that received plain drinking water (Water) during pregnancy. A fresh supply of the appropriate type of water was provided to each group every other day. Following parturition, all mice received plain drinking water.

In a previous study we established that mice receiving 2 mg/ml L-DOPA plus 0.025% ascorbic acid in drinking water showed a 50% increase in the dopamine content of the fetal brain and a significant decrease in cell proliferation on E13 compared to mice receiving ascorbic acid alone or plain drinking water (Ohtani et al., 2003). Administration of 1 mg/ml L-DOPA plus 0.025% ascorbic acid in drinking water did not produce significant changes in those measurements (Ohtani et al., 2003). Therefore, we used 2 mg/ml L-DOPA in the present study. We did not administer a peripheral dopamine decarboxylase inhibitor (to prevent oxidation of L-DOPA in the gastrointestinal system) or a catechol-O-methyl transferase inhibitor (to prevent dopamine methylation) to the mice, as the increase in dopamine content of the fetal brain produced without the use of these inhibitors was sufficient to alter cell proliferation (Ohtani et al., 2003). We established that the water intake by mice in the three groups was comparable (~13 ml/pregnant mouse/day; averaged over 8 days from the 11th day of gestation to the day before parturition). Other studies have also employed this L-DOPA treatment paradigm to induce elevated dopamine levels in the fetal brain (Marien et al., 1984; Porter et al., 1999; Schmidt et al., 1996).

In the experiments described below, we used at least 2–3 male mice each from at least 3 litters for each prenatal treatment group to control for inter- and intra-litter variation. The total numbers of mice used from each treatment group is indicated separately for each experiment.

2.3. Conditioned place preference (CPP) and locomotor sensitization

We perfromed an unbiased CPP assay as follows. We used a 3-chamber place preference apparatus (Med Associates Inc., St. Albans VT, USA) made of 2 equal sized (16.8 × 12 cm) preference chambers connected by a central chamber (7.2 × 12 cm) to perform an unbiased CPP assay. The apparatus is outfitted with sliding guillotine-style doors between each compartment, and photo-beams wired to a computer to record animal location and motor activity. The central chamber has a smooth, grey floor while each preference chamber is either white with a mesh floor or black with a bar floor.

On the first day of experimentation (Preconditioning) mice were placed in the central chamber for a 1-min (60 s) habituation period with the sliding doors closed, followed by a 20-min (1200 s) period of free exploration throughout the whole apparatus. Time spent in each chamber was recorded, and mice were returned to their home cage. To control for apparatus bias, the following criteria are applied to the preconditioned data. Mice that do not fit the criteria are excluded from the study. First, any mouse that spends more than 500 s of the 20-min test period in the central grey chamber is excluded. Of the mice that qualify, the time spent in the grey chamber is subtracted from 1200 (the total test time in seconds), and divided by 2 to give a "half-time" (equal to the amount of time the mouse would spend in each chamber, black or white, if it had absolutely no preference). The half-time is multiplied by 0.2 and this value is used to give a range (upper and lower limits) of allowable time a mouse can spend in the black or white chamber. If the time the animal spends in one chamber exceeds the upper or lower limits of the range then the animal is assumed to have an apparatus bias and is removed from the study.

Mice are then randomly assigned to a treatment group (cocaine or saline control). In a series of preliminary studies, we performed a dose-response analysis using 5, 10 or 15 mg/kg (i.p.) cocaine in Water (control) group of mice. These studies showed that 15 mg/kg cocaine produced the most reliable and consistent CPP compared to that produced by the other doses. Therefore, we used 15 mg/kg dose in the subsequent experiments. On days 2–4 (conditioning) mice are given an injection of cocaine (15 mg/kg; i.p.) or saline and confined to either the black or white chamber for 20 min. Mice are then returned to their home cages. After 4 h a saline injection (0.01 ml/g body weight) is given, and the mice are confined to chamber opposite to that in which they were given cocaine. On the final day (test day) mice are again placed in the central chamber with the doors closed for a 1-min habituation. The doors are raised and the mice are allowed to move freely about the chamber. Time spent in each chamber is recorded.

Preference is determined as the time spent in the drug-paired chamber on the test day minus time spent in the drug-paired chamber on the preconditioning day. All data are collapsed across drug-paired chambers because initial experiments showed that the preference obtained by drug-pairing in the black or in the white chamber was indistinguishable.

Locomotor activity was measured in transparent, 28 by 28 cm boxes with 16 photocells on each side equipped with the hardware and software required for data collection (MedAssociates, Georgia, Vermont). Mice were placed individually in the test box for a 15-min habituation period, removed from the box, injected with cocaine (15 mg/kg; i.p.) or saline and returned to their respective box for 30 min during which time activity was monitored.

The data for conditioned place preference and locomotor activity assays were analyzed by ANOVA. Post-hoc Fisher's PLSD test was used for comparison between the treatment groups. The numbers of mice used (n) in each treatment group for the conditioned place preference assays was: L-DOPA – 7, ASC – 8 and Water – 8. For the locomotor activity assays, 8 mice were used from each treatment group.

2.4. In vivo microdialysis

P21 male mice from all three prenatal treatment groups (L-DOPA, ASC acid and Water) were implanted with guide cannulae in the striatum. P60 male mice from the L-DOPA and ASC groups were implanted with guide cannulae in the caudate putamen or nucleus accumbens. Implantation of the guide cannulae was performed under ketamine/xylazine anesthesia (120 mg/kg i.p. dosed by the ketamine component) in a stereotaxic frame (Kopf Instruments, Tujunga, CA). Body temperature was monitored and maintained between 37° and 38 °C on a heating pad. The scalp was cleansed with povidone iodine, incised, and hemostasis was achieved by electrical cautery if necessary. Two 0.9 mm burr holes were drilled in the skull. Stereotaxic coordinates relative to bregma were derived for the caudate putamen (striatum) and nucleus accumbens from the mouse brain atlas (Paxinos and Franklin, 2001). The stereotaxic coordinates were as follows. P60 caudate putamen: A/P +0.5 mm, D/V +4.5 mm, M/L ±1.85 mm; P60 nucleus accumbens: A/P +1.3, D/V +2.9 (15°), M/L ±2.0; P21 striatum: A/P +0.5 mm; D/V +4.4 mm; M/L ±1.8 mm. Biocompatible polyurethane guide cannulae (5.0 mm in length, CMA Microdialysis, North Chelmsford, MA, model CMA/7) were secured to the skull with dental acrylic resin, and 5.0 mm stainless-steel stylets were inserted until the time of experiments.

Incisions were treated with 2.5% topical xylocaine gel and antibiotic ointment, and closed with 3–0 silk. Following surgery, mice were housed singly with *ad libitum* access to food and water, and allowed a minimum of 4 days of post-surgical recovery prior to all experiments. Thus, the P26 mice were cannulated on P21 or P22 (because some litters were weaned on P22 rather than P21) and the P60 mice were cannulated on P56. One stainless-steel/polycarbonate concentric microdialysis probe (240 μm outer diameter, 7.0 mm total length, 1.0 mm exposed tip length; CMA Microdialysis) was stereotaxically inserted into a guide cannula under ketamine/xylazine anesthesia (80 mg/kg) on the evening prior to each experiment and secured with dental acrylic resin. Only the right hemisphere was targeted in each case. Animals were then returned to a circular acrylic experimental cage, where they were allowed to recover overnight with *ad libitum* access to food and water. Each mouse underwent probe insertion through each guide cannula for only one experiment. Probes were perfused at a rate of 1.0 μl/min with artificial cerebrospinal fluid (aCSF) of the following composition (in mM): NaCl 125; KCl 2.5; NaH2PO4 0.5; Na2HPO4 5.0; MgC12 1.0; CaC12 1.2; D-glucose 5.0; pH = 7.4 and filter-sterilized through a 0.22 μm cellulose acetate membrane. Probes were perfused overnight prior to the day of the experiment with aCSF through a singlechannel fluid swivel (Instech Laboratories, Plymouth Meeting, PA). For all microdialysis experiments, samples were collected every 10 min in awake, behaving mice.

In vitro probe recovery tests were performed prior to the in vivo studies in order to assure that the probe was functioning well. The data were not corrected for probe recovery, which should not be different between the different prenatal treatment groups. Of note, we have utilized the perfusion rate of 1.0 μl/min in our previous in vivo experiments also (Malanga et al., 2009) that generated values comparable to those reported by others (Fumero et al., 1994; Martin-Fardon et al., 1997). Moreover, we report our dialysis values as percentage of baseline, which avoids concerns regarding incomplete probe recovery, which should not be different among the 3 prenatal treatment groups. In the experiments that report only the baseline data also the probe recovery should be comparable among the 3 prenatal treatment groups.

Following the microdialysis experiments, the mice were decapitated, brains were dissected and snap-frozen in liquid nitrogen. Coronal sections (30 μm) of the brains were cut on a cryostat and processed for cresyl violet staining. The location of the probe in the caudate putamen or nucleus accumbens was verified based on the probe track in the histological sections. Microdialysis data from mice in which the probes were in the wrong location were discarded.

Data from 18 mice out of a total of 21 mice each at P26 and P60 were included in the analysis. In the P26 group, probe placements were incorrect in the 3 mice that were excluded from the study. In the P60 group one mouse died during the experiment and the probe placements were found to be incorrect in the other two. The data were analyzed by ANOVA. Post-hoc Tukey-multiple comparisons test was used for comparison between the treatment groups.

2.5. Analysis of tissue concentration of dopamine and its metabolites in the striatum and midbrain

We analyzed monoamine neurotransmitter and metabolite concentrations in P14, P21 and P60 mice. The mice were anesthetized by inhalation of isoflurane and decapitated. The brains were dissected and snap-frozen in liquid nitrogen. In representative cases from each of the 3 prenatal treatment groups at P21, we weighed the mice prior to anesthesia and recorded weights of their brains prior to freezing to determine if the prenatal treatment produced changes in these measurements.

The frozen brains were sectioned on a cryostat up to the level of the anterior forceps, where a 1.0 mm coronal slab was cut. The striatum (caudate putamen and nucleus accumbens) and ventral midbrain were visually identified and tissue punches were obtained from each region bilaterally using a biopsy punch. We could not reliably distinguish between the caudate putamen and nucleus accumbens in the P14 brains. Therefore, to maintain uniform sampling across the 3 age groups, we collected samples of the striatum combining the caudate putamen and nucleus accumbens. Samples from the right and left sides for each region from each brain were pooled for further analysis. The punched out tissue samples were weighed and homogenized by ultrasonification in buffer (150 mM phosphoric acid; 0.2 mM ethylenediamine tetra acetic acid [EDTA]). Crude homogenates were centrifuged at 14,000 rpm for 20 min and the supernatant was filtered through a 0.22 μm cellulose acetate membrane.

In each tissue sample, we analyzed dopamine (DA) and its metabolites 3,4 dihydroxyphenylacetic acid (DOPAC) and 3-methoxy-4-hydroxyphenyl acetic acid (homovanillic acid, HVA). Quantification of monoamines was performed isocratically at room temperature by HPLC through a model MD-150 analytical column (C18 3.2 mm × 150 mm; 3 μm particle size; 120 Å pore size) at a flow rate of 0.6 ml/min with electrochemical detection (model 5600A Coulochem II electrochemical detector; model 5011 analytical cell with palladium reference electrode; model 5020 guard cell; ESA, Chelmsford MA) at the following potentials: guard cell +250 mV; pre-oxidation −100 mV; detector +240 mV. The mobile phase consisted of 90 mM NaH_2PO_4 (monohydrate); 50 mM citric acid (monohydrate); 1.7 mM 1-octanesulfonic acid; 50 μM EDTA; and 10% (vol/vol) acetonitrile; pH = 3.00 (phosphoric acid); filtered through a 0.22 μm cellulose acetate membrane, and degassed by ultrasonification. Under these conditions the retention time for monoamines was 3–11 min and the limit of detection was below 1.5 fmol. Monoamine levels were quantified by external standard curve calibration using CoulArray software (v.1.12; ESA). Monoamine standards (1.0 μM, 0.5 μM and 0.1 μM) were run at the beginning and end, and after every eighth sample for re-calibration during each experiment.

The data were analyzed by ANOVA. Differences between groups were analyzed by using Tukey post-hoc multiple comparison test. We used 6 mice from each age group and from each prenatal treatment (Fig. 1).

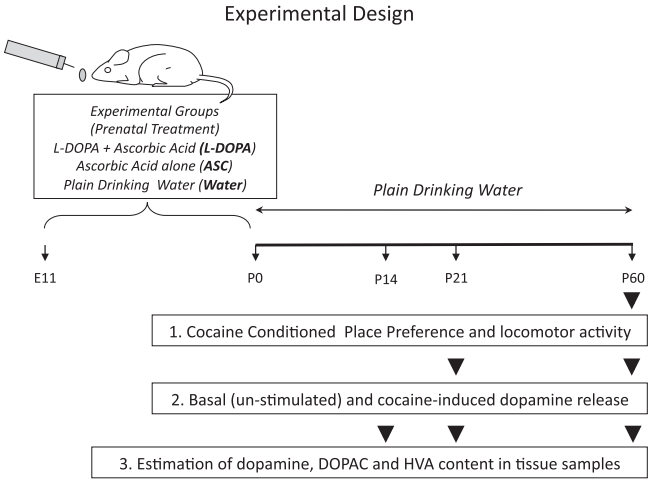

Fig. 1. A diagrammatic representation of the experimental design. Timed-pregnant CD1 mice were assigned to 3 experimental groups beginning on the 11th day of pregnancy: The mice in the L-DOPA group received drinking water containing L-DOPA (2 mg/ml) plus ascorbic acid (0.025%); the mice in the Ascorbic Acid group received drinking water containing 0.025% ascorbic acid; whereas the mice in the Water group received plain drinking water (i.e. drinking water without additives). On the day of parturition and during the nursing period the mice in all the 3 groups received the plain drinking water. Each of the 3 groups of mice was subjected to 3 types of experiments: 1. On postnatal day 60 (P60), we performed cocaine conditioned place preference and cocaine-induced locomotor activity assays; 2. On P26 and P60, we performed in vivo microdialysis to assay basal (un-stimulated) and cocaine-induced dopamine release in the striatum (P26), caudate putamen (P60) and nucleus accumbens (P60); and 3. On P14, P21 and P60 we analyzed tissue concentrations of dopamine, DOPAC and HVA in the striatum (caudate putamen and nucleus accumbens) and the midbrain using HPLC.

3. Results

3.1. Conditioned place preference and locomotor activity

Mice in the ASC and Water groups showed significant preference for the cocaine-paired chamber on the test day compared to the preconditioning period (Fig. 2A). However, mice in the L-DOPA group did not show significant differences in preference between the saline- and cocaine-paired chambers (Fig. 2A). ANOVA revealed significant main effects of the prenatal treatment ($F = 3.82$, $p < 0.05$) and post-hoc analysis revealed that the L-DOPA group showed significantly lower place preference compared to the ASC and Water groups ($p < 0.05$ for both comparisons). The ASC and Water groups did not differ significantly ($p > 0.05$) from each other. There was no statistically significant difference among the 3 groups of mice in locomotor activity following acute cocaine exposure (Fig. 2B; ANOVA, treatment effects, $F = 0.610$, $p > 0.05$).

3.2. Cocaine-induced dopamine release

Since cocaine place preference differed between L-DOPA and the two control groups (ASC and Water) we examined cocaine-induced dopamine release in the 3 groups of mice in the striatum at P26 and caudate putamen and nucleus accumbens at P60 by using in vivo microdialysis. The rationale for performing the microdialysis

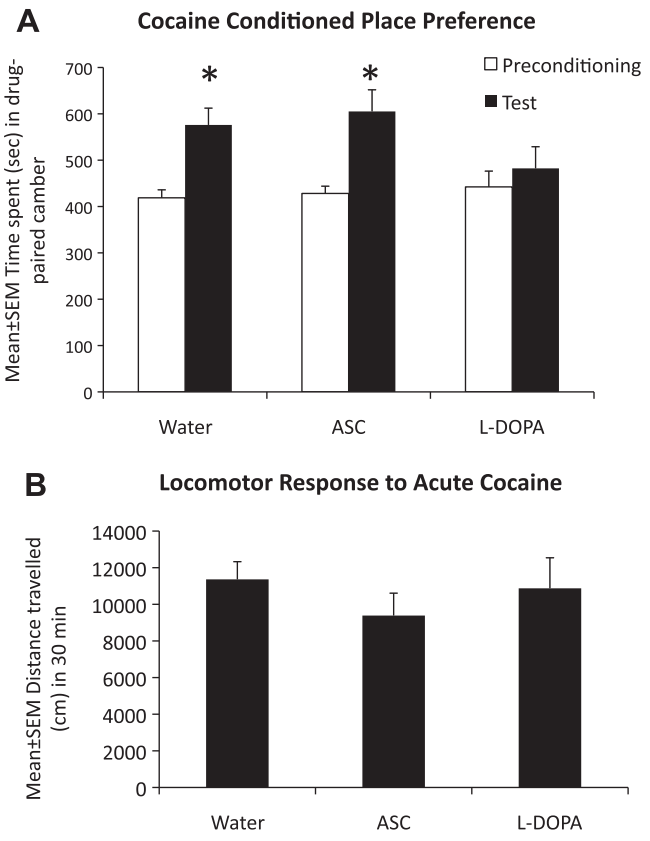

Fig. 2. Cocaine conditioned place preference (A) and cocaine-induced locomotor activity (B) assays were performed in P60 mice exposed to drinking water containing L-DOPA plus ascorbic acid (L-DOPA), ascorbic acid alone (ASC) or plain drinking water (Water) from embryonic day 11 until the day of birth. The mice in the Water and ASC groups showed significant place preference by spending significantly longer periods of time in the cocaine-paired chamber during the test session compared to the pre-conditioning session (A). However, the mice in the L-DOPA group did not show place preference (A). Mice in all the 3 groups showed comparable locomotor activity upon a single intraperitoneal cocaine administration (B). * = $p < 0.05$; ANOVA and Post-hoc Fisher's PLSD test.

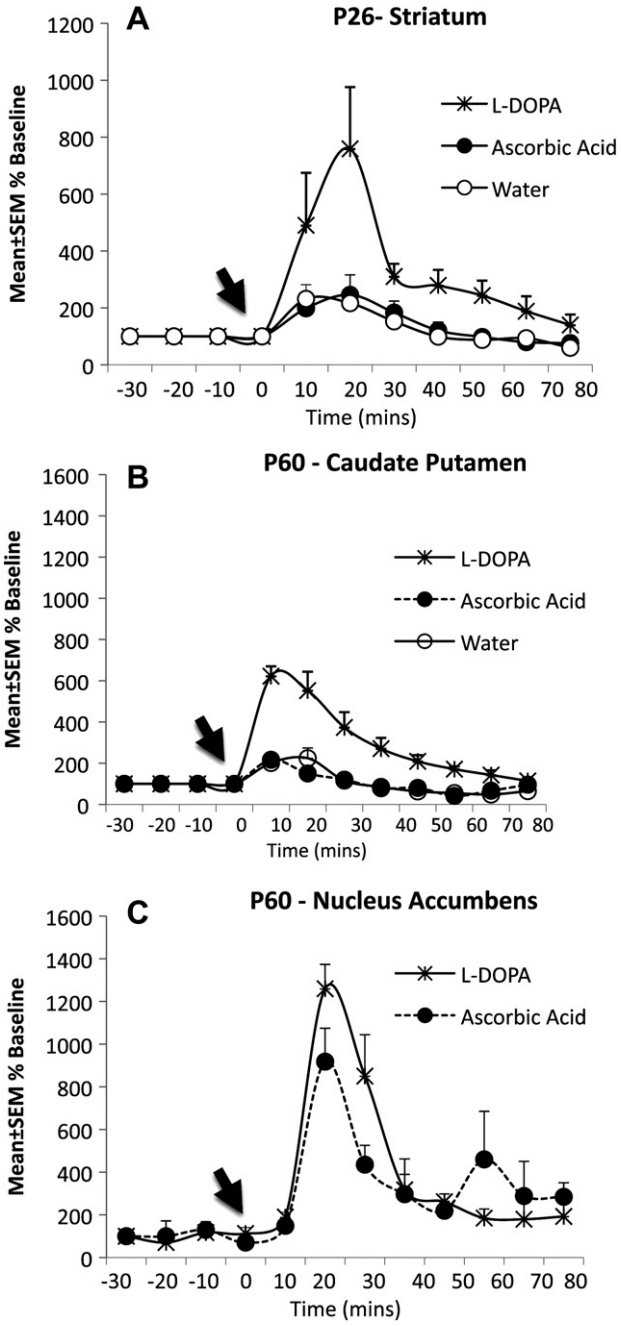

Fig. 3. Microdialysis and HPLC analysis were performed in postnatal day 26 (P26) and P60 mice exposed to drinking water containing L-DOPA plus ascorbic acid (L-DOPA), ascorbic acid alone (ASC) or plain drinking water (Water) from embryonic day 11 until the day of birth. Microdialysis probes were placed under stereotaxic guidance in the caudate putamen in awake, P26 and P60 mice and the nucleus accumbens in awake, P60 mice. The analysis in the nucleus accumbens was performed only in the L-DOPA and ASC groups. Samples were collected every 10 min for HPLC analysis. Following 30 min of sample collection for analysis of basal levels of dopamine and metabolites, cocaine (15 mg/kg) was administered intraperitoneally (arrows in A, B and C) and samples were collected at 10 min intervals for an additional 1 h. The data showed that mice in all 3 groups showed a significant increase in dopamine release for ~30 min following the cocaine administration in the caudate putamen on P26 (A) and P60 (B) and nucleus accumbens on P60 (C). The increase in cocaine-induced dopamine release was significantly higher (* $p < 0.05$; ANOVA) in the L-DOPA group in the caudate putamen on P26 (A) and P60 (B). However, in the nucleus accumbens the dopamine release was not significantly different in the L-DOPA group compared to the ASC group.

experiments was that release of dopamine from ventral midbrain dopaminergic axon terminals in the striatum is typically associated with the action of drugs of abuse such as cocaine (Carelli, 2004; Wise, 1996). The P26 caudate putamen analysis was included for correlation with our earlier data on neuron and glial cell numbers in this region at P21 in the same 3 experimental groups used here (McCarthy et al., 2007).

We found that dopamine release was induced in the caudate putamen of all 3 groups of mice within 10 min of cocaine administration at P26 and P60 (Fig. 3A, B). ANOVA revealed significant effects of prenatal treatment at P26 ($F = 3.86$; $p < 0.05$) and P60 ($F = 5.51$; $p < 0.01$). At both ages, the dopamine release was higher in the L-DOPA group than the ascorbic acid and water groups. Multiple comparisons analysis did not reveal statistically significant differences between the 3 experimental groups at P26. However, at P60, the L-DOPA group showed significantly higher dopamine release compared to the ASC and Water controls ($p < 0.05$).

We could not reliably target the nucleus accumbens in the P21 mice for placement of the guide cannula and microdialysis probe because of the small size of this brain region. Therefore, we did not use the P21 mice and used only the P60 mice for analysis of dopamine release in the nucleus accumbens. Moreover, since the ASC and Water groups did not differ significantly in conditioned place preference (Fig. 2A), locomotor activity (Fig. 2B) or cocaine-induced dopamine release in the caudate putamen on P26 (Fig. 3A) or P60 (Fig. 3B), we used the ASC group and not the Water group as vehicle control for dopamine release in the P60 nucleus accumbens.

We found significant increases in dopamine release following cocaine injection in the L-DOPA and ASC groups in the nucleus accumbens at P60 (Fig. 3C). However, there was no statistically significant difference in the magnitude of dopamine release between the two groups. In other words, the L-DOPA and ASC groups showed similar increases in dopamine release in the nucleus accumbens following the cocaine exposure.

Basal levels of dopamine release were similar among the 3 groups of mice in the caudate putamen at P26 and P60 (P21: $F = 0.26$, $p > 0.05$; P60: $F = 0.04$; $p > 0.05$; Fig. 4A, B). However, the L-DOPA group showed significantly higher basal dopamine levels in the nucleus accumbens at P60 compared to ASC controls ($F = 41.62$; $p < 0.0001$; Fig. 4C).

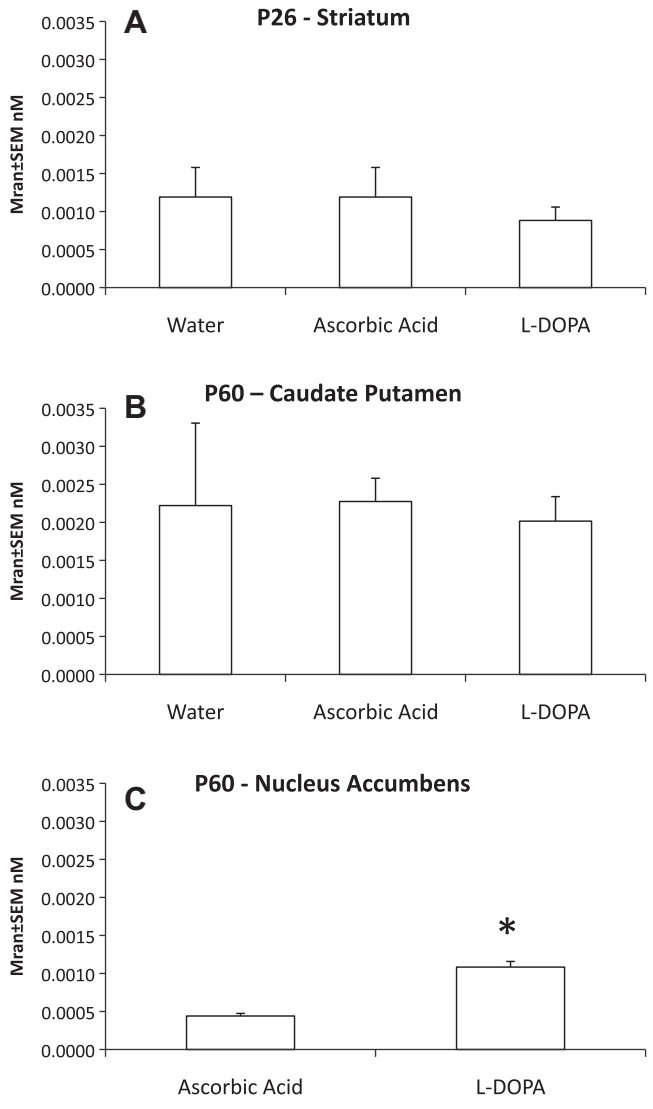

Fig. 4. Dopamine release prior to stimulation with cocaine (basal release) was analyzed in postnatal day 26 (P26) and P60 mice exposed to drinking water containing L-DOPA plus ascorbic acid (L-DOPA), ascorbic acid alone (ASC) or plain drinking water (Water) from embryonic day 11 until the day of birth. The analysis was performed by HPLC in samples collected from the caudate putamen on P26 (A) and P60 (B) and the nucleus accumbens on P60 (C) in awake mice by using microdialysis. The analysis in the nucleus accumbens was performed only in the L-DOPA and ASC mice. There was no statistically significant difference in dopamine release among the 3 groups of mice in the caudate putamen on P26 or P60. However, the dopamine release was significantly higher in the nucleus accumbens of L-DOPA mice compared to the ASC mice (C). * = $P < 0.05$.

The prenatal treatment only affected dopamine release and did not produce statistically significant effects on DOPAC or HVA levels (basal or cocaine-induced) in the caudate putamen or nucleus accumbens at P26 or P60 (data not shown).

When we compared the levels of cocaine-induced dopamine, DOPAC and HVA in the caudate putamen in each prenatal treatment group between P26 and P60, none of the comparisons showed statistically significant difference (data not shown). Similarly, cocaine-induced dopamine, DOPAC or HVA levels did not show statistically significant difference between the caudate putamen and nucleus accumbens at P60, the only age for which nucleus accumbens data were collected.

3.3. Dopamine and metabolite content of the striatum

We examined tissue concentration of dopamine and its metabolites DOPAC and HVA in the striatum and midbrain in the 3 groups of mice at P14, P21 and P60. We found that dopamine content increased from P14 to P60 in the striatum and midbrain in all 3 groups. In the striatum, overall differences among the 3 groups in the dopamine content were statistically significant (Fig. 5A; $F = 22.5$; $p < 0.001$; $n = 6$). Developmental changes (as a function of age) and changes due to experimental treatment in dopamine content were statistically significant (developmental stage: $F = 72.81$; treatment: $F = 14.1$; both cases $p < 0.001$ and $n = 6$). However, the interaction between developmental stage and experimental treatment with respect to dopamine content was not statistically significant ($F = 1.65$; $p > 0.05$). When developmental stage was considered, comparisons between P60 and P21; P21 and P14 as well as P60 and P14 showed significant differences with the older ages showing higher dopamine content than the younger ages (Fig. 5A). When experimental treatment was considered, the L-DOPA group showed significantly higher dopamine content compared to the ASC and Water groups (Fig. 5A).

The DOPAC content showed significant overall differences among the 3 groups ($F = 12.07$; $p < 0.001$; $n = 6$). Both developmental stage and experimental treatment produced significant effects on DOPAC content (developmental stage, $F = 38.84$, $p < 0.001$; treatment, $F = 4.34$, $p < 0.05$). However, the interaction between the two factors with respect to the DOPAC content was not statistically significant ($F = 2.65$; $p > 0.05$). Comparison among the 3 experimental groups showed that the only significant difference was between L-DOPA and ASC groups with the L-DOPA group showing significantly lower DOPAC levels. Comparison among the 3 developmental stages showed significant differences between P60 and P21 and P60 and P14, with the P60 group showing higher DOPAC levels (Fig. 5B).

Analysis of HVA content showed significant overall effects ($F = 3.38$; $p < 0.05$) due to significant effects of developmental stage ($F = 10.05$; $p < 0.001$). The experimental treatment did not produce significant effects ($F = 1.55$; $p > 0.05$) on DOPAC content nor was the interaction between experimental treatment and developmental stage significant ($F = 1.95$; $p > 0.05$). Comparisons among the 3 age groups showed significant differences between P60 and P14 as well as P60 and P21, with P60 showing the lowest HVA content, followed by P21 and P14 (Fig. 5C).

3.4. Dopamine and metabolite content of the midbrain

In the midbrain, analysis of dopamine content showed significant overall effects ($F = 6.29$; $p < 0.001$). The effects of developmental stage ($F = 4.65$) and experimental treatment ($F = 14.48$) on the dopamine content as well as the interaction between the two factors ($F = 3.01$) were statistically significant (experimental treatment, $p < 0.001$; developmental stage and interaction,

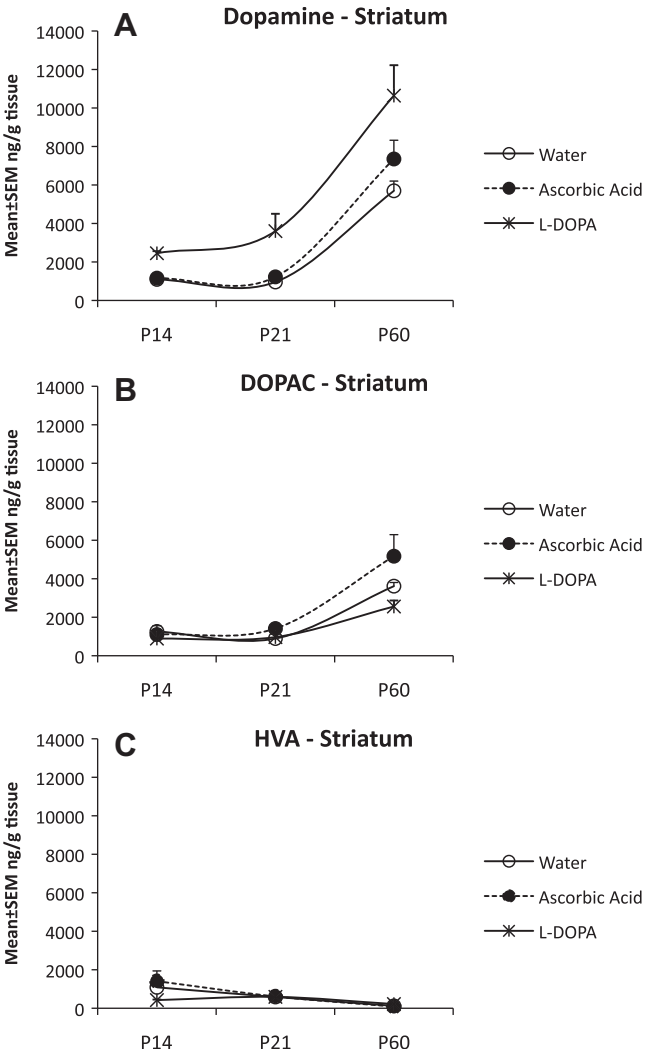

Fig. 5. Tissue concentrations of dopamine (A) and its metabolites 3,4 dihydroxyphenylacetic acid (DOPAC; B) and 3-methoxy-4-hydroxyphenyl acetic acid (homovanillic acid, HVA; C) were measured in the striatum (caudate putamen plus nucleus accumbens) by HPLC in postnatal day 14 (P14), P21 and P60 mice exposed to drinking water containing L-DOPA plus ascorbic acid (L-DOPA), ascorbic acid alone (ASC) or plain drinking water (Water) from embryonic day 11 until the day of birth. The prenatal treatment significantly affected the concentrations of dopamine and DOPAC, with the L-DOPA group showing higher dopamine levels and lower DOPAC levels. The HVA concentration was unaffected by the prenatal treatment. The developmental stage also produced significant effects on all 3 measurements such that dopamine and DOPAC concentrations increased while the HVA concentration decreased with age.

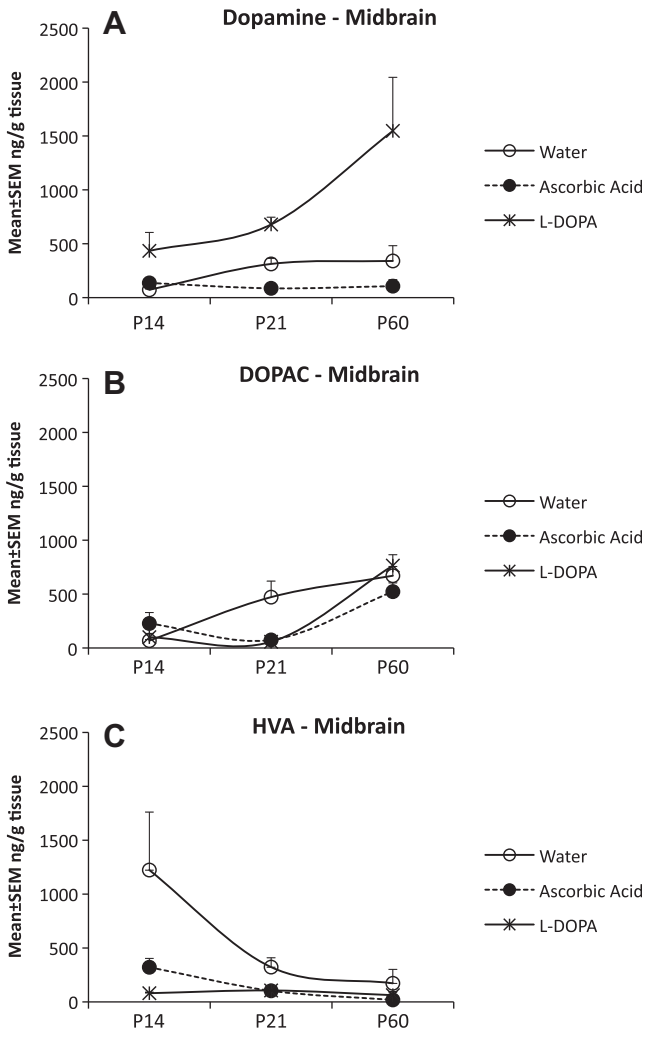

Fig. 6. Tissue concentrations of dopamine (A) and its metabolites 3,4 dihydroxyphenylacetic acid (DOPAC; B) and 3-methoxy-4-hydroxyphenyl acetic acid (homovanillic acid, HVA; C) were measured in ventral midbrain by HPLC in postnatal day 14 (P14), P21 and P60 mice exposed to drinking water containing L-DOPA plus ascorbic acid (L-DOPA), ascorbic acid alone (ASC) or plain drinking water (Water) from embryonic day 11 until the day of birth. The prenatal treatment significantly affected the concentrations of dopamine and HVA, with the L-DOPA group showing higher dopamine and lower HVA concentrations. Developmental stage also significantly affected all 3 measurements such that dopamine and DOPAC concentrations increased while the HVA concentration decreased with age.

$p < 0.05$). When the 3 experimental groups were compared, the dopamine content of the L-DOPA group was significantly higher than those of the ASC and Water control groups ($p < 0.05$; Fig. 6A). When the 3 age groups were compared only the difference between P60 and P14 was statistically significant, with the P60 group showing higher dopamine content than the P14 group (Fig. 6A).

Analysis of the ventral midbrain DOPAC content showed significant overall effects ($F = 11.22$; $p < 0.001$) with developmental stage producing significant effects ($F = 33.22$, $p < 0.001$). The interaction between developmental stage and experimental treatment was statistically significant ($F = 4.79$; $p < 0.001$) although the main effects of the experimental treatment on DOPAC content were not significant ($F = 2.08$; $p > 0.05$). None of the pair-wise comparisons among the 3 experimental groups was statistically significant. When the 3 developmental stages were compared, the differences between P60 and P21 as well as P60 and P21 were statistically significant, with the older age groups showing higher DOPAC content.

Analysis of ventral midbrain HVA content showed significant overall effects ($F = 3.63$; $p < 0.01$), effects of the experimental treatment ($F = 5.76$; $p < 0.01$) and developmental stage ($F = 4.39$; $p < 0.05$). The interaction between experimental treatment and developmental stage with respect to HVA content was not significant ($F = 2.19$; $p > 0.05$). Comparison of differences between the experimental groups showed that the L-DOPA group had significantly lower HVA content compared to the ASC and Water controls (Fig. 6C). Comparison among the 3 age groups showed significant differences between P60 and P14 groups, with the P14 groups showing higher HVA content (Fig. 6C).

We measured body and brain weights from representative mice in each of the 3 prenatal treatment groups at P21. Mean ± SEM values for body weight (g) were: L-DOPA ($n = 9$) 16.75 ± 0.85; ASC

($n = 11$) 16.18 ± 0.91; Water ($n = 9$) 16.71 ± 0.57. Mean ± SEM values for brain weight were: L-DOPA ($n = 9$) 0.41 ± 0.01; ASC ($n = 11$) 0.42 ± 0.01; Water ($n = 9$) 0.43 ± 0.01. There was no statistically significant effect of the prenatal treatment on either measurement (body weight: $F = 0.0.07$, $p > 0.05$; brain weight: $F = 1.07$; $p > 0.05$).

4. Discussion

Our data show that elevated dopamine levels in the fetal brain produced by administration of L-DOPA to the mother in the drinking water lead to persisting increases in dopamine content of the striatum and midbrain; increased cocaine-induced dopamine release in the caudate putamen and decreased cocaine conditioned place preference. The L-DOPA treatment also led to increased basal (un-stimulated) dopamine release in the nucleus accumbens but did not alter it in the caudate putamen. Furthermore, the L-DOPA group did not show significant changes in cocaine-induced locomotor activity or cocaine-induced dopamine release in the nucleus accumbens.

These data suggest that prenatal L-DOPA exposure, which produces significant increases in the dopamine content of the fetal brain (Ohtani et al., 2003), leads to lasting neurochemical and behavioral changes. The neurochemical changes (i.e. increases in basal or cocaine-induced dopamine release) are region-specific because they did not affect the caudate putamen and nucleus accumbens uniformly. The behavioral changes also showed selectivity in that cocaine place preference was decreased while cocaine-induced locomotor activity was not affected. These neurochemical and behavioral changes may reflect differential effects of prenatal L-DOPA exposure on midbrain dopaminergic pathways projecting to the caudate putamen *versus* those projecting to the nucleus accumbens and frontal cortex. Equally likely, the differential changes may reflect differential effects on the nucleus accumbens core and shell compartments, each of which is believed to mediate different aspects of (motor *versus* cognitive) dopaminergic behaviors (Cadoni et al., 2000; Ito et al., 2000).

A major consequence of the prenatal L-DOPA exposure is the loss of cocaine place preference (Fig. 2). Our microdialysis data show that acute exposure to cocaine produces significant increases in dopamine release in the caudate putamen and nucleus accumbens in the L-DOPA group, just as it does in the control groups. In fact, the cocaine-induced dopamine release in the caudate putamen was nearly 8 times higher in the L-DOPA group than the other groups (Fig. 3). Therefore, attenuated dopamine release may not be an explanation for the lack of cocaine place preference in the L-DOPA group with the caveat that we only measured cocaine-induced dopamine release following a single administration of cocaine. Our microdialysis data showed that basal or un-stimulated dopamine release was significantly higher in the nucleus accumbens of the L-DOPA group compared to the ASC (control) group (Fig. 4). It is possible that the elevated basal dopamine release somehow increased the threshold for inducing cocaine place preference in the L-DOPA group. However, this possibility has not been tested directly yet. Moreover, since we did not examine whether higher doses of cocaine (higher than the 15 mg/kg used here) could produce place preference or whether place preference could be induced by other drugs (e.g. amphetamine, heroin) or stimuli (e.g. food), the precise mechanisms underlying the loss of cocaine place preference in the L-DOPA group remain obscure (Palmiter, 2008; Zombeck et al., 2008).

We do not know if repeated cocaine exposure, such as that which occurred during the conditioned place preference assays, differentially alters the magnitude of dopamine release in the caudate putamen and/or nucleus accumbens of the L-DOPA group compared to the controls and whether such differences contributed to the lack of cocaine place preference. Moreover, it is possible that the L-DOPA group may not differ from the control groups in other operant measures of reward such as drug self-administration or intracranial electrical self-stimulation despite the differences shown here in measures employing classical (Pavlovian) conditioning such as CPP (Malanga et al., 2007, 2008).

Another explanation for the differential effects of prenatal exposure to L-DOPA on CPP *versus* locomotor activity may be that the gestational L-DOPA treatment can produce different effects on the core *versus* shell components of the nucleus accumbens. The accumbens core is associated with motor behaviors (neo-striatum mediated behaviors) whereas the accumbens shell is associated with behaviors such as addiction (limbic-striatum behaviors) leading to the suggestion that the shell may be a part of the "extended amygdala" complex (Cadoni et al., 2000; Ito et al., 2000). Additional studies will be required to address this potentially interesting issue because our present data cannot resolve structural or neurochemical differences between the core and shell of the nucleus accumbens.

Adult mice exposed prenatally to cocaine also show significant decrease in cocaine's potency in conditioned place preference assays without changes in locomotor activity (Malanga et al., 2007, 2009), just as adult mice exposed prenatally to L-DOPA did in the present study. Early developmental exposure (occurring in the postnatal period) to another stimulant methylphenidate in rats also decreased cocaine place preference in adulthood (Carlezon et al., 2003; Mague et al., 2005). Thus, decreased cocaine place preference at adulthood may be a consequence common to developmental exposure to L-DOPA, cocaine or methylphenidate although each drug produces elevation of brain dopamine content via different mechanisms (Kubrusly et al., 2007; Levitt, 1998; Meyer et al., 1993; Wang et al., 1995). Thus, elevated dopamine levels during the prenatal period, regardless of the specific nature of the contributing molecular mechanism, appear to produce lasting changes in biochemical and behavioral characteristics. This finding underscores the potential role of developmental dopamine imbalance in contributing to neuropsychiatric disorders of multiple etiologies, including schizophrenia, autism spectrum, and attention deficit hyperactivity.

Analysis of tissue dopamine, DOPAC and HVA contents showed significant developmental changes in these parameters in the midbrain and striatum. Although the dopamine and DOPAC contents increased from P14 to P60 in both the brain regions, the HVA content decreased significantly. The L-DOPA treatment resulted in permanent increases in dopamine content in the striatum as well as the midbrain. The L-DOPA treatment also altered dopamine metabolism and turnover in that it decreased DOPAC content in the striatum (Fig. 5) and increased the HVA content in the midbrain (Fig. 6) at P60.

Another significant insight emerging from our data is that the neurochemical and behavioral changes produced by prenatal L-DOPA exposure occur in the absence of readily discernible structural changes. For example, we showed that the same L-DOPA treatment protocol as that used here did not produce significant changes in the total numbers of neurons or glial cells in the frontal cortex or the striatum (McCarthy et al., 2007). The only cellular change produced by the L-DOPA exposure was subtle changes in frontal cortical and striatal neuronal numbers that could be revealed by BrdU birth-dating methods (McCarthy et al., 2007). Thus, elevated dopamine levels in the fetal brain lead to significant neurochemical and behavioral changes without producing significant changes in brain structure. Therefore, the prenatal L-DOPA exposure model may be paradigmatic for neuro-functional developmental disorders such as schizophrenia that are characterized by

significant functional changes despite a lack of structural changes such as mal-positioning of neurons. The behavioral changes in these neuro-functional disorders are associated with neurochemical changes such as decreased neurotrophin levels that contribute to altered synaptic function (Durany et al., 2001). Similarly, there may also be postsynaptic changes in the biochemical and molecular responsivity to dopaminergic signaling as a result of the developmental presynaptic changes we have identified.

L-DOPA is predominantly used for the treatment of Parkinson's disease. Pregnancy is rare in Parkinson's disease, whose symptoms usually appear in women after the child-bearing age. However there are case reports of pregnant women receiving L-DOPA treatment for Parkinson's Disease (Hagell et al., 1998). The general consensus is that L-DOPA treatment during pregnancy is safe because L-DOPA is not believed to be teratogenic. However, our data for the first time highlight the potential for long-term neurochemical and behavioral changes in the offspring following gestational exposure to L-DOPA.

In summary, our data show that prenatal L-DOPA exposure produces persisting behavioral and neurochemical changes. Since these changes occur in the absence of readily discernible anatomical changes in the brain, the prenatal L-DOPA exposure model may be relevant for developmental dopaminergic disorders that manifest as functional rather than structural disorders.

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