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Elevated Dopamine Levels During Gestation Produce Region-specific Decreases in Neurogenesis and Subtle Deficits in Neuronal Numbers

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Abstract

Dopamine levels in the fetal brain were increased by administering the dopamine precursor 3,4-dihydroxy-L-phenylalanine (L-DOPA) to pregnant mice in drinking water. The L-DOPA exposure decreased bromodeoxyuridine (BrdU) labeling in the lateral ganglionic eminence and frontal cortical neuroepithelium but not medial or caudal ganglionic eminences. The regional differences appear to reflect heterogeneity in precursor cells' responses to dopamine receptor activation. Relative numbers of E15 generated neurons were decreased at postnatal day 21 (P21) in the caudate-putamen, nucleus accumbens and frontal cortex but not globus pallidus in the L-DOPA group. TUNEL labeling did not show significant differences on P0, P7 or P14 in the caudate-putamen or frontal cortex, suggesting that cell death was not altered. Although virtually all cells in the P21 brains that were labeled with the E15 BrdU injection were NeuN-positive, stereological analyses showed no significant changes in total numbers of NeuN-positive or NeuN-negative cells in the P21 caudate-putamen or frontal cortex. Thus persisting deficits in neuronal numbers were evident in the L-DOPA group only by birth-dating analyses and not upon gross histological examination of brain sections or analysis of total numbers of neurons or glia. One explanation for this apparent discrepancy is that L-DOPA exposure decreased cell proliferation at E15 but not at E13. By E15, expansion of the neuroepithelial precursor pool is complete and any decrease in cell proliferation likely produces only marginal decreases in the total numbers of cells generated. Our L-DOPA exposure model may be pertinent to investigations of neurological dysfunction produced by developmental dopamine imbalance.

Keywords

ganglionic eminence; dopamine; neurogenesis; striatum; caudate-putamen; frontal cortex

1. Introduction

Dopamine and its receptors appear in the developing brain early in the embryonic period before the onset of synaptogenesis [5,14,20,39,53]. Therefore, a role for dopamine in brain development that may be independent of its role at the synapse in the mature brain appears likely [24,25,28,38]. Activation of dopamine receptors influences cell proliferation in the lateral ganglionic eminence (LGE) and the neuroepithelium of the frontal cortex in embryonic

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mice [39,43,72,73] and in the subventricular zone and hippocampal dentate gyrus in the adult mice [18,22,64]. Dopamine receptor activation also influences GABA neuron migration from the basal forebrain to the cerebral cortex [13] and differentiation of cortical and striatal neurons [50,56].

An interesting implication of dopamine's role in cell proliferation and differentiation is the likelihood that even a transient imbalance in dopamine levels in the developing brain could translate into lasting impairments of the structure and function of the mature brain. Dopamine imbalance can occur following maternal intake of drugs such as cocaine that cross the placental barrier and interfere with dopaminergic signaling mechanisms in the fetal brain [1,19,23,28,35,36,52,67]. Neuro-functional disorders such as schizophrenia, autism spectrum disorder and attention deficit hyperactivity disorder also appear to be associated with imbalance in dopamine and other neurotransmitters in the developing brain [10,34,47,66]. Some of the drugs (e.g. dopamine receptor agonists, antagonists, methylphenidate etc) that are used in the treatment of these disorders also can produce imbalance in dopamine levels in the brain.

In the present study, we show that elevated dopamine levels in the fetal brain induced by oral administration of the dopamine precursor 3,4-dihydroxy-L-phenylalanine (L-DOPA) to pregnant mothers can decrease BrdU labeling in the LGE and frontal cortical neuroepithelium in the E15 brain leading to subtle but significant, alterations in relative numbers of E15-generated neurons in the cerebral cortex, caudate-putamen and nucleus accumbens at P21. An interesting feature of these alterations is that they are not evident upon gross histological examination of brain sections or analysis of total numbers of neurons or glia. Instead, fine-grained neuronal birth dating analyses are required to reveal the changes. In this regard, the gestational L-DOPA exposure model may be paradigmatic for disorders such as schizophrenia, autism or attention deficit hyperactivity that are known to be associated with developmental dopamine imbalance but not with gross neuropathology or changes in total neuronal or glial numbers in any one brain region consistently.

2. Results

We examined the effects of elevated dopamine levels in the fetal brain induced by administering L-DOPA to pregnant CD1 mice in drinking water. Three groups of mice were created depending on the type of drinking water provided (Fig. 1): "L-DOPA" group received L-DOPA (2 mg/ml) plus Ascorbic Acid (0.025); "ASC" group received ascorbic acid alone (0.025%); and "WATER" group received plain drinking water (Fig. 1). Ascorbic acid was used to retard oxidation of L-DOPA in the water [32,44,50]. The treatments began on the 11th day of pregnancy and lasted until parturition. On embryonic day 13 (E13; day of conception = E0) or E15, a single intraperitoneal injection of bromodeoxyuridine (BrdU) was administered to the pregnant mice. Four types of analyses were performed on fetal and postnatal brains to assess the effects of the experimental treatments. 1. Cell proliferation in the forebrain neuroepithelial domains was analyzed on E13 and E15. For these analyses, embryos were sacrificed 2.0 hr after the BrdU injection and BrdU labeling index (LI; BrdU-labeled cells ÷ total cells per unit area) was analyzed in forebrain neuroepithelial domains. 2. In other experiments, on postnatal day 21 (P21; day of birth = P0), BrdU-NeuN double labeling was performed in different regions of the forebrain of mice exposed to BrdU on E15. 3. Cell death was analyzed in the embryonic and postnatal brains using TUNEL histochemistry. 4. Stereological methods were used to estimate total numbers of NeuN-positive and NeuN-negative cells in the caudate putamen and frontal cortex on P21.

Technical considerations

We established in an earlier study [39] by measurement of the brain dopamine content by using HPLC that oral administration of L-DOPA to pregnant mice resulted in significant elevation

of the fetal brain dopamine level. In that study mice receiving 2 mg/ml L-DOPA plus 0.025% ascorbic acid in drinking water from the 10th day of gestation showed a 50% increase in the dopamine content of the fetal brain on E13 (3 days after L-DOPA administration began) compared to mice receiving ascorbic acid alone or plain drinking water. Administration of 1mg/ml L-DOPA plus 0.025% ascorbic acid in drinking water did not produce significant changes in those measurements [39] demonstrating a dose-dependent effect of the oral L-DOPA administration. The L-DOPA administration paradigm used in the present study is identical to that used previously except that the L-DOPA treatment began on the 11th day of gestation in the present study. Based on the results of the previous study, we expect that fetal brain dopamine levels would have increased by at least 50% in the L-DOPA group in the present study by E14. Our analyses of cell proliferation in the embryonic brain were performed on E15, when fetal brain dopamine levels would be significantly elevated in the L-DOPA group. 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. We established that the water intake by mice in the three groups was not significantly different (~13ml consumption/pregnant mouse/day). One other study have also employed similar L-DOPA treatment paradigm to induce elevated dopamine levels in the fetal brain [46], although it was concerned with the effects on cell proliferation. We began the L-DOPA treatment on E11 as it represents the time of onset of neurogenesis in the dorsal and basal forebrain neuroepithelial domains of the mouse [54,58].

Cell proliferation

We examined BrdU LI (BrdU-labeled cell number ÷ total cell number per unit area) in the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE), caudal ganglionic eminence (CGE) and the neuroepithelium of the frontal cortex on E13 and E15. The mean ±SEM BrdU LI values in each neuroepithelial domain for each of the 3 experimental groups are shown in Table 1. When the data were analyzed by ANOVA, we did not find statistically significant differences in BrdU LI among the 3 experimental groups in any of the regions at E13. However, ANOVA showed significant differences in the LGE ($p < 0.01$) and the frontal cortex ($p < 0.05$) and not in the MGE or CGE at E15. Tukey's Multiple Comparisons test showed that the differences in the E15 LGE were due to significant decreases in the BrdU LI in the L-DOPA group compared to the WATER and ASC groups (Table 1). In the frontal cortical neuroepithelium, the significant difference in BrdU LI was due to a significant reduction in the BrdU LI in the L-DOPA group compared to the ASC group (Table 1).

BrdU-TH double labeling in the MGE and CGE

We explored the possibility that the lack of significant differences in BrdU LI in the MGE and CGE may be due to lack of dopaminergic innervation of these neuroepithelial domains at E15. We found that TH immunoreactive profiles were in close proximity to BrdU-labeled cells near the lateral ventricular border both in the MGE and CGE at E15 (Fig 2 E, F). Our earlier work had shown that TH-positive axons are in close proximity to BrdU-labeled cells in the LGE and the frontal cortical neuroepithelium [39,43]. Thus, presumptive dopaminergic axons enter all the neuroepithelial domains analyzed here. We emphasize that lower power images show robust TH immunoreactivity only within striatal differentiating fields (Fig 2 C, D), which contain postmitotic cells. Only at higher magnifications (e.g. 40X objective) TH-labeled axon terminals and growth cones were seen entering the neuroepithelial domains of the MGE and CGE and come in close proximity to BrdU-labeled cells (Fig 2 E, F). It was the case also in the LGE and the frontal cortical neuroepithelium at E15 [39,43].

Effects of D1-receptor activation on BrdU LI in the E15 MGE and CGE

Since D1-like receptor binding sites predominate over D2-like binding sites in the embryonic forebrain [20,39,45,49] and since the reduction in BrdU LI in the E15 LGE and frontal cortical neuroepithelium in the L-DOPA group are similar in direction and magnitude to the reduction in BrdU LI in the same regions produced by the selective activation of the D1-receptor [39, 43], we explored the possibility that in contrast to the LGE and frontal cortex, the D1-receptor activation in the E15 MGE and CGE somehow did not lead to alterations in the BrdU LI. The Mean $\pm$ SEM values of BrdU LI following administration of the selective D1-receptor agonist SKF 81297 (10 or 20 mg/kg) or saline are shown in Table 2. ANOVA did not reveal significant differences in this measurement for MGE or CGE among the experimental groups ($p>0.05$), indicating that D1-receptor activation did not alter BrdU LI in these regions, although the BrdU LI was decreased significantly in the LGE and frontal cortical neuroepithelium following selective activation of the D1 receptor (Table 2) (Popolo et al., 2004).

Analysis of BrdU LI in the brains of P21 mice

We examined the BrdU LI in the caudate putamen, nucleus accumbens, globus pallidus and frontal cortex in P21 mice that had received a single BrdU injection on E15. The goal was to determine if the L-DOPA-induced decreases in the BrdU LI in the LGE and the neuroepithelium of the frontal cortex in the E15 mice translated into significant decreases in the BrdU LI at P21 in brain regions that receive cells from those two precursor populations. The caudate-putamen and nucleus accumbens receive cells from the LGE [33,57]. The frontal cortex receives its projection neurons from the frontal cortical neuroepithelium and interneurons from the MGE, CGE and to a lesser extent from the LGE [2,3,26,33,37,57,69, 71]. The globus pallidus likely receives its cells from the MGE.

We examined BrdU LI in the dorso-medial and ventro-lateral regions of the caudate-putamen of the 3 groups at P21 (Fig. 3) to take into account regional variations in the distribution of E15-generated neurons [6,15,31]. The mean $\pm$ SEM values are shown in Table 3. ANOVA showed significant differences in the BrdU LI in both the dorso-medial and ventro-lateral regions of the caudate-putamen ($p<0.01$ for both) among the 3 experimental groups. Tukey's Multiple Comparisons test (Table 3) showed that the differences in both the dorso-medial and ventro-lateral regions were due to a decrease in the BrdU LI in the L-DOPA group compared to the ASC group (both $p<0.01$). The BrdU LI in the ASC group was significantly higher compared to the WATER group in both the dorso-medial ($p<0.05$) and the ventro-lateral ($p<0.01$) regions (Table 3).

ANOVA revealed a significant difference in the mean $\pm$ SEM values of BrdU LI among the 3 experimental groups in the nucleus accumbens (Table 4). Tukey's test revealed that the difference was due to a significant decrease ($p<0.01$) in the BrdU LI in the L-DOPA group compared to the ASC group. There were no significant differences in the BrdU LI between the ASC and WATER groups.

The mean $\pm$ SEM values for BrdU LI in the P21 globus pallidus were not significantly different among the 3 experimental groups (L-DOPA: 0.06 ± 0.03 ; ASC: 0.09 ± 0.03 ; WATER: 0.09 ± 0.01).

BrdU LI in the P21 frontal cortex was analyzed initially for the cellular layers of the cortex (layers II to VI; Fig. 3C). The majority of the BrdU-labeled cells were located in layers II–III, corresponding to the superficial (i.e. towards the pial surface) 25% of the cortical gray matter (Fig 3C), as expected [4,7,8,11,58]. Therefore, we analyzed the BrdU LI separately for the upper 25% (layers II–III) and lower 75% of the gray matter (layers V–VI) as well as for all cellular layers (II–VI). The mean $\pm$ SEM values for BrdU LI are shown in Table 5. ANOVA

showed that there was a significant difference ($p < 0.01$) in the BrdU LI among the 3 experimental groups when the data were analyzed for layers II–VI. The difference was due to a reduction in the BrdU LI in the L-DOPA group compared to the ASC group (Table 5), whereas there were no significant differences between ASC and WATER groups.

When the data were analyzed by ANOVA for layers II–III only (superficial 25%), where the majority of the BrdU-labeled cells were found, significant differences ($p < 0.05$) were found in this measurement among the 3 groups. The difference was due to a significant ($p < 0.05$) decrease in the BrdU LI in the L-DOPA group compared to the ASC group (Table 5). ANOVA did not reveal significant differences among the groups in the BrdU LI in layers V–VI (lower 75%).

We also compared the numerical density of basic fuchsin-labeled nuclei among the 3 experimental groups in the caudate-putamen (Table 3), nucleus accumbens (Table 4) and the frontal cortex (Table 5). There were no significant differences in this measurement among the 3 groups in any of the regions examined (ANOVA, $p > 0.05$).

Analysis of BrdU-NeuN double-labeling

To determine if the cells labeled with BrdU in the P21 brains following the E15 BrdU injections were neuronal or non-neuronal cells, we counted BrdU-labeled cells that were also positive for the neuronal marker NeuN (Fig. 2 A, B). We performed this analysis in the caudate-putamen and the frontal cortex, two regions that showed a significant effect of L-DOPA on BrdU LI at P21. We found that 90–99% of the BrdU-labeled cells were also NeuN-labeled in these two regions (Table 6) and the 3 experimental groups did not show significant differences in this measurement (ANOVA; $p > 0.05$).

Stereological Estimates of total numbers of NeuN-positive and NeuN-negative cells in the caudate putamen and frontal cortex

Since BrdU LI was reduced in the P21 caudate-putamen and frontal cortex of the L-DOPA group and since the BrdU-positive cells were predominantly NeuN-positive, we examined if the total numbers of NeuN-positive cells was decreased in the P21 caudate-putamen or frontal cortex of the L-DOPA group. We used an unbiased stereological technique, the optical fractionator to estimate the total numbers of NeuN-positive and NeuN-negative cells in the caudate-putamen and frontal cortex (Fig. 4). The data are shown in Table 7. ANOVA did not reveal significant differences among the 3 experimental groups in total numbers of NeuN-positive or NeuN-negative cells in any of the regions analyzed. Our estimates of total cell numbers in the caudate putamen and frontal cortex are consistent with estimates by others [29,40,48]. However, we were concerned that the penetration of the NeuN or the secondary antibody into the histological sections during the immunohistochemistry procedure may be uneven or incomplete (i.e. the antibodies may have penetrated up to only ~5–10 μ m from surface) producing variability in NeuN labeling among the three groups and therefore introducing errors in the data analysis [51]. To determine if NeuN-positive cells were evenly distributed throughout the depth of the section among the three experimental groups, we compared the distribution of NeuN-positive profiles through the thickness of the optical disector in each of the 3 groups. NeuN-labeled profiles were present throughout the thickness and ANOVA showed that the distribution was not significantly different ($P > 0.05$) among the 3 experimental groups (Figure 4D).

Analysis of cell death

The changes in the BrdU LI among the three experimental groups at P21 may be a direct result of changes in cell proliferation as reflected in changes in the BrdU LI at E15 and/or a result of changes in cell survival. We examined if cell survival was significantly different among the 3

experimental groups by using TUNEL histochemistry to analyze the incidence of apoptosis (Fig. 2). We counted TUNEL-positive profiles in the LGE, MGE and CGE and the frontal cortical neuroepithelium in the E15 brains. We limited the analysis to the caudate-putamen and frontal cortex at the postnatal intervals (P0, P7 and P14), as relatively extensive data on the timetable of developmental cell death are available for these two regions [16,63,65]. The available data indicate that developmental cell death is more or less completed by P14 in both these regions. We analyzed only the dorso-medial region of the caudate-putamen and only the superficial layers of the frontal cortex, as the BrdU LI in both these regions showed significant differences among the 3 experimental groups. Mean $\pm$ SEM values of percent TUNEL labeling for each of the regions at each age are shown for the 3 experimental groups in Table 8. We did not find statistically significant differences in the incidence of TUNEL+ profiles among the three groups in any of the regions by ANOVA ($p>0.05$) at any of the ages examined - E15, P0, P7 or P14.

3. Discussion

We show that increased dopamine content of the fetal brain produced by L-DOPA administration to pregnant mice decreases BrdU LI in the LGE and the frontal cortical neuroepithelium at E15 and produces deficits in the relative numbers of E15-generated neurons in the frontal cortex, caudate-putamen and nucleus accumbens at P21. BrdU LI in the MGE or CGE did not show significant changes suggesting regional specificity of the effects. The BrdU LI was not significantly altered by the L-DOPA treatment at E13 in any of the regions analyzed. Stereological analyses of total numbers or neuronal and non-neuronal cells or comparison of numerical densities of basic fuchsin labeled nuclei did not reveal significant differences among the experimental groups, suggesting that the deficits in E15-generated neurons revealed by BrdU birth dating studies were not sufficient to alter the overall cell numbers.

It is intriguing that only the LGE and frontal cortical neuroepithelium showed significant changes in BrdU LI on E15 due to L-DOPA exposure. We addressed potential explanations for this difference. We examined if LGE, MGE, CGE and frontal cortex received endogenous dopaminergic inputs in the embryonic period. Our TH-BrdU immunohistochemical data confirm that TH-positive, presumptive dopaminergic terminals are in close proximity to BrdU-labeled proliferating cells in the MGE and CGE, just like in the LGE and frontal cortical neuroepithelium [39,43]. In a previous study, we showed that mRNA for all 5 dopamine receptor subtypes were present in the LGE, MGE, CGE and the frontal cortical neuroepithelium as early as by E12 [5]. Therefore, differences in dopaminergic innervation or expression of dopamine receptors are unlikely to be the basis for the regional differences in decreased BrdU LI. An explanation for the differences was suggested by our finding that the reduction in BrdU LI in the E15 LGE and frontal cortical neuroepithelium in the present study is similar in direction and magnitude to the decreases in BrdU LI produced by pharmacological activation of dopamine D1-like receptors both in vivo and in vitro [39,43]. Therefore, differences in the way the D1-receptor activation influences proliferation of precursor cells might underlie the differences in BrdU LI changes between the LGE and frontal cortical neuroepithelium *versus* MGE and CGE. This appears to be the case. When E15 embryos were exposed to the D1-receptor agonist SKF 81297 the BrdU LI in the MGE or CGE did not show significant changes. In an earlier study we showed that the same dose of SKF 81297 (10 or 20 mg/kg) produced significant decreases in BrdU LI in the LGE and the frontal cortical neuroepithelium in E15 mice [43]. Therefore, it is likely that for reasons not yet clear, D1-like receptor activation does not alter cell proliferation in the MGE or CGE in the same manner as it does in the LGE or frontal cortical neuroepithelium.

There is a significant decrease in BrdU LI in the P21 caudate putamen, nucleus accumbens and the frontal cortex following a BrdU injection on E15 in the L-DOPA group. This decrease is

consistent with the decrease in BrdU LI in the E15 LGE and frontal cortical neuroepithelium in that these two neuroepithelial domains give rise to cells of the caudate putamen, nucleus accumbens and frontal cortex. The BrdU LI in the P21 globus pallidus following a BrdU injection on E15 did not show significant changes among the 3 experimental groups. The globus pallidus derives its cells from the MGE and as mentioned earlier MGE did not show significant changes in BrdU LI on E15.

Ascorbic acid group showed significant increases in BrdU LI in the P21 caudate putamen compared to the water group. Previous reports showed that ascorbic acid can influence proliferation and differentiation of CNS progenitor cells both in vivo and in vitro [27,32,42, 55,70]. Our data appear to be consistent with these reports. Ascorbic acid did not significantly alter BrdU LI on E15 in the LGE or any other neuroepithelial domain in this study or in our earlier studies [39,43]. Nor did it significantly alter cell death, as revealed by TUNEL labeling in the present study. Therefore, the ascorbic acid-induced increase in BrdU LI in the caudate putamen at P21 cannot be explained by changes in cell proliferation. Neither the nucleus accumbens nor frontal cortex showed significant effects of ascorbic acid treatment on BrdU LI at P21.

By using BrdU and NeuN double labeling, we established that virtually all of the cells labeled with the E15 BrdU injection were neurons in the frontal cortex and the caudate putamen. These data would suggest that the total numbers of neurons in the caudate putamen, nucleus accumbens and frontal cortex would be decreased in the L-DOPA group. However, our stereological analyses did not show significant differences in NeuN-positive cell numbers in the caudate putamen or frontal cortex at P21 (we did not analyze the nucleus accumbens) among the three experimental groups. Even in the upper layers of the frontal cortex where the BrdU LI differences were most pronounced, we did not find significant changes in cell numbers by stereology. At first glance the P21 BrdU LI data and the stereological estimates appear to be irreconcilable. However, our E13 BrdU LI data help resolve the apparent inconsistency. Since there was no significant difference in the BrdU LI in the LGE or frontal cortical neuroepithelium (or any of the other regions) among the 3 experimental groups at E13, the effects of L-DOPA administration on neurogenesis appear to have begun around E15 and not earlier. By E15 the cell output from of the telencephalic neuroepithelium has increased such that more than 50% of the daughter cells produced following a round of cell division exit the cell cycle [54,60]. In other words, cell divisions do not contribute to expansion of the founder population. Therefore, changes in neuroepithelial cell proliferation/output occurring on or after E15 are likely to have only small effects on the total numbers of cells produced by the neuroepithelium in contrast to changes occurring at earlier stages, which would deplete the founder population and result in significant deficits in total numbers of cell produced [60,61]. Our earlier studies showed that dopamine receptors are expressed in the E13 forebrain, including the LGE and frontal cortical neuroepithelium and that dopamine receptor activation can alter the BrdU LI in the E13 LGE [39]. Therefore, the lack of changes in the BrdU LI on E13 is unlikely to be due to lack of functional dopamine receptors at that age.

These data raise interesting points about the type of “pathology” produced by the elevated dopamine levels during gestation in our mouse model. In this model although neurogenesis is altered on E15 and (presumably after E15 as dopamine levels continue to remain high), persisting changes in neuronal numbers are evident only by using BrdU birth-dating methods: total neuronal or glial cell numbers did not change. These data underscore the subtle nature of neuronal changes and suggest that the gestational L-DOPA exposure model presented here may be paradigmatic for developmental neuro-functional disorders such as schizophrenia, attention deficit or prenatal drug abuse. The rationale for this suggestion is that all these conditions are associated with developmental dopamine imbalance and significant cognitive impairment without significant changes in total numbers of neurons or glia in a specific brain

region. Whether the changes produced by the L-DOPA exposure in our model translate into functional or behavioral changes remains to be determined.

The increases in dopamine level produced by the L-DOPA exposure model described here likely differ from the alterations in dopaminergic transmission produced by exogenously administered dopaminergic drugs. It is an important distinction in that exogenously administered drugs can activate dopamine receptors globally whereas L-DOPA-induced elevation of dopamine level likely has regional effects - only in those brain regions that receive dopaminergic input. In addition, the cell and molecular mechanisms involved in the elevation of dopamine levels also differ between the L-DOPA exposure model and other models of drug-induced increases in dopamine. The latter (e.g. cocaine) tend to increase extracellular dopamine level by blocking dopamine re-uptake while L-DOPA increases dopamine synthesis. We recognize also that although the L-DOPA administration ceased on the day of birth in our study, the L-DOPA-induced elevation of brain dopamine levels may continue into the postnatal period because of residual L-DOPA in the offspring or the nursing mother.

In conclusion, our data show that elevation of dopamine levels in the fetal brain can reduce cell proliferation in the embryonic telencephalon in a region-specific manner. The regional selectivity of the effects appears to be due to regional differences in the activity of the D1-receptor rather than regional differences in dopaminergic innervation. The decreased cell proliferation translates into lasting deficits in relative numbers of E15 generated neurons in the caudate-putamen, nucleus accumbens and frontal cortex. However, a conventional analysis such as estimation of total numbers of neurons or glia in the caudate putamen or frontal cortex fails to illustrate these deficits highlighting the potential problem associated with identifying the neuropathology in neuro-functional disorders associated with developmental dopamine imbalance. Whether the deficit in E15-generated neurons reported here are sufficient to produce functional alterations in the caudate-putamen, nucleus accumbens or frontal cortex remains to be seen. If it did, our gestational L-DOPA exposure model could mimic neuro-functional disorders associated with developmental dopamine imbalance.

4. Experimental Procedures

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 are in full compliance with institutional and NIH guidelines.

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 (2mg/ml) was dissolved in drinking water containing 0.025% ascorbic acid (Sigma). Ascorbic acid prevents oxidation of L-DOPA in the water [32,44,50]. Control groups received drinking water containing 0.025% ascorbic acid alone or drinking water without additives. Thus, we created 3 groups of mice (Fig. 1): 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 daily. At the time of changing the water supply, we measured the volume of water remaining in each bottle to obtain a rough estimate of daily water consumption. Following parturition, all mice received plain drinking water. Therefore, the oral administration of L-DOPA or ascorbic acid lasted only during gestation (Fig. 1).

Analysis of cell proliferation

Mice in each of the three groups were administered a single injection of the S-phase marker BrdU (Sigma; 50 µg/g body weight, i.p.) on E13 or E15 (Fig 1). For analysis of cell proliferation in the E13 or E15 brain, the dams were anesthetized (Ketamine, 50 mg/kg body weight and Xylazine, 10 mg/kg body weight, i.p.) 2.0 hr after the BrdU injection and the embryos were removed. Although we received timed-pregnant mice from the vendor, we ascertained the age of each embryo by examination of the external morphological features [21,62]. Embryos (or entire litters) that did not fulfill the criteria for E13 or E15 were discarded. The embryos judged to be of the correct age were decapitated and their heads were fixed by immersion in 70% ethanol. The brains were removed from the skull within an hour of decapitation, immersed in 70% ethanol overnight at room temperature and processed for paraffin-wax histology. We cut 4µm-thick sections of the paraffin-embedded brains in the coronal plane on a rotary microtome and processed the sections for BrdU immunohistochemistry using a monoclonal anti-BrdU antibody (Becton Dickinson, Mountain View, CA) and cobalt-nickel enhancement of diaminobenzidine reaction product [9,39,43,59]. The sections were stained with 0.1% aqueous basic fuchsin. In these preparations, BrdU-labeled nuclei appear black and non-BrdU-labeled nuclei appear reddish pink [39,43].

We calculated BrdU labeling index (BrdU LI; BrdU-labeled/all nuclei/bin) by counting BrdU-labeled and unlabeled nuclei within a 120X240µm² sector of the LGE, a 120X120 µm² sector of the MGE, 120X120µm² sector of the CGE and a 100X100µm² sector of the neuroepithelium of the frontal cortex. The sector for the LGE was divided into twenty bins (each bin was 120X12µm²), the sectors for each of the MGE and CGE into ten bins (each bin was 120X12µm²) and the sector for the cortical neuroepithelium also into ten bins (each bin was 100X10µm²). The sectors were aligned such that bin 1 was at the ventricular surface, with its long axis parallel to the ventricular border. The BrdU LI was calculated separately for each bin. The different sizes of the sectors and bins represent different sizes of the neuroepithelium in the different regions. The neuroepithelium of the LGE extends over a larger area compared to the other neuroepithelial domains analyzed here, due to the large size of its subventricular zone [9,39,43,54]. The different sizes also correspond to the sizes used in our previous work, facilitating comparisons across studies [9,12,39,43,54].

Analysis of the distribution of cells labeled with BrdU on E15 in the brains of P21 mice

Following the BrdU injection on E15, some of the dams from each experimental group were allowed to litter; the offspring were anesthetized on P21 (Ketamine, 50 mg/kg body weight and Xylazine, 10 mg/kg body weight, i. p.) and perfused via the heart with 70% ethanol. The brains were post-fixed in 70% ethanol overnight and processed for paraffin-wax histology. Coronal 4µm-thick sections were cut and BrdU immunohistochemistry and basic fuchsin staining were performed, as described earlier. BrdU LI was calculated in the frontal cortex, caudate-putamen, nucleus accumbens, and globus pallidus (Fig. 3A, B). In the frontal cortex the entire gray matter from layer II to VI was divided into 10 X 100 µm² bins and the BrdU LI was calculated for each bin (Fig 3B). In the caudate-putamen, the BrdU LI was calculated separately for the dorso-medial and ventro-lateral regions (Fig. 3A) within 240 X 240 µm² sampling sectors. The BrdU LI was calculated within 240 X 240 µm² sectors in the nucleus accumbens and globus pallidus (Fig. 3A).

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) analysis

We examined cell death in the LGE, MGE, CGE and frontal cortical neuroepithelium at E15 and in the caudate-putamen and frontal cortex at P0, P7 and P14 in each of the three experimental groups. The mice were anesthetized with halothane (Isoflurane) and decapitated. The brains were removed and snap-frozen in isopentane cooled to -40°C in liquid nitrogen.

The frozen brains were sectioned on a cryostat in the coronal plane at a thickness 12 μm and processed for TUNEL histochemistry according to manufacturer's instructions (Roche, Purchase, NY). The sections were stained with propidium iodide (Sigma- Aldrich, St Louis, MO) to label nuclei, coverslipped with Gelmount and examined under a fluorescence microscope. The total number of nuclei (i.e. propidium iodide labeled) and TUNEL + profiles were counted within defined areas of each region of interest in 8 non-consecutive sections from each brain.

BrdU and NeuN double labeling

We performed BrdU and NeuN double-label immunohistochemistry on sections of P21 brains. Mice from the 3 experimental groups were anesthetized as described earlier and perfused through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. The brains were removed and post-fixed in the same fixative at 4°C for 2 hr and sectioned on a Vibratome in the coronal plane at a thickness of 100 μm . The sections were processed free-floating for double-label immunohistochemistry using a rat monoclonal anti-BrdU antibody (1:100; Abcam, Cambridge, MA) and a mouse monoclonal anti-NeuN antibody (1:100; Chemicon, Temecula, CA). We examined the sections under a laser confocal microscope (Zeiss, Pascal LSM) and counted BrdU- labeled, NeuN-labeled and BrdU+NeuN double-labeled cells. The cells were counted within defined sectors of layers II–III in the frontal cortex and the dorso-medial and ventro-lateral regions of the caudate-putamen. The counts were converted to number of cells per 1000 μm^2 .

BrdU and tyrosine hydroxylase (TH) double-immunohistochemistry

We administered 3 BrdU injections (50 $\mu\text{g/g}$ body weight, i.p.) to dams carrying E15 mice. The injections were spaced 3 hr apart, beginning at 9 AM. We chose the 3 hr interval because BrdU administered to the mother is available for uptake in the fetal brain for at least 3 hr [17, 59]. The mice were anesthetized as described above and the embryos were removed and decapitated 0.5 hr after the third BrdU injection. Thus, the embryos were exposed to BrdU for a total of 6.5 hr. This 6.5 hr BrdU labeling period was chosen so as to label a larger proportion of precursor cells distributed throughout the VZ and SVZ [9] than would have been possible with the shorter (e.g. 2.0 hr) labeling intervals used in the BrdU LI analyses. The embryonic heads were fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer. The brains were removed and stored in the same fixative at 4°C for 18–24 hr. Vibratome sections of the brains were cut at a thickness of 60 μm in the coronal plane. Double-labeling immunohistochemistry for BrdU and TH was performed using a polyclonal TH antibody (Chemicon, Temecula, CA; diluted 1:1000), a monoclonal anti-BrdU antibody (Becton Dickenson, Mountain View, CA; 1:75 dilution) and fluorescent secondary antibodies (Alexa conjugated donkey anti-rabbit IgG and donkey anti-Mouse IgG; Jackson ImmunoResearch, Westgrove, PA). The sections were mounted on glass slides and coverslipped using a water-soluble mounting medium. The sections were examined in a Zeiss LSM laser confocal microscope.

Administration of dopamine D1-like receptor agonist SKF 81297

We administered 2 injections of the D1-like receptor agonist SKF 81297 (RBI/Sigma, 10 mg/kg or 20 mg/kg; i.p.), or saline (control) to pregnant mice carrying E15 embryos. The injections were spaced 3 hr apart. BrdU (50mg/kg; i.p.) was administered 1 hr after the second SKF 81297 injection. The mother was anesthetized 2 hr after the BrdU injection and the embryos were removed, decapitated and the embryonic heads were immersed in 70% ethanol. Thus, the embryos were exposed to SKF 81297 for a total of 6 hrs and BrdU for the final 2 hrs. The brains were removed from the heads and stored in 70% ethanol at room temperature for 18 to 24 hr and then embedded in paraffin wax. BrdU immunohistochemistry was performed on

sections of the brain as described earlier. We calculated the BrdU LI in the MGE and CGE also as described earlier. An earlier study had used the same experimental design to demonstrate that selective activation of the dopamine D1-receptor decreased 2.0 hr BrdU LI in the LGE and the frontal cortical neuroepithelium on E15 [43].

Stereology

Mice from the 3 experimental groups were anesthetized on P21 (as described above) and perfused through the heart with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. The brains were removed and post-fixed in the same fixative at 4°C overnight and cryoprotected in 30% sucrose at 4°C overnight. The brains were frozen in powdered dry ice, mounted on a sliding microtome stage with the caudal end down and coronal sections were cut at 35µm thickness. Every section that contained the caudate-putamen was collected in rostral-to-caudal serial order in phosphate buffer (0.1 M, pH 7.2). A stereotaxic mouse brain atlas [41] was used to identify the structures. The most rostral section that contained the caudate-putamen corresponded to Figure 17 (stereotaxic coordinates inter-aural = 5.5 mm and bregma = 1.70 mm) and the most caudal section to Figure 48 (stereotaxic coordinates: inter-aural = 1.74 mm and bregma = -2.06 mm) in the atlas. The sections collected also contained the primary and secondary motor areas of the cortex.

Based on a roll of die every 6th section of the series was chosen for stereological analyses. Preliminary analyses using every 4th, 6th or 8th section established that every 6th section produced reliable estimates of cell numbers in the caudate-putamen as well as the frontal cortex. Each selected section was processed free floating for NeuN immunohistochemistry using a mouse monoclonal anti-NeuN antibody (1:100; Chemicon, Temecula, CA) and DAB as the chromagen. The sections were stained with 0.1% aqueous cresyl violet (Sigma), dehydrated in ethanol, cleared in xylene and coverslipped with Permount mounting medium.

An unbiased stereological technique, the optical fractionator [29,30,68], was used to estimate the total number of NeuN-positive and NeuN-negative cells in the caudate-putamen and frontal cortex [29,30]. The equipment consisted of a light microscope (Nikon E2000) connected to a cool CCD camera (Microfire; Optronics, Goleta, CA), motorized X-Y stage (Ludl Electronics Products, Hawthorne, NY), z-axis indicator (MT12 microcator; Heidenhain, Traunreut, Germany), and a computer running Stereo Investigator software (Microbrightfield, Inc., Colchester, VT). The caudate-putamen was identified using anatomical landmarks (corpus callosum, external capsule, lateral ventricle, globus pallidus, and anterior commissure). In the rostral sections, the ventral striatum was excluded from analysis by a line drawn from the ventral tip of the lateral ventricle to the dorsal border of the piriform cortex [29,30]. Frontal agranular cortex was identified by using histological landmarks (absence of layer IV). Distinguishing between primary and secondary motor areas was difficult. Therefore, we refer to the two areas collectively as frontal cortex.

Outlines of the caudate-putamen or frontal cortex were drawn at 2X magnification in each section. The frontal cortex was divided into upper (layers II and III) and lower (layers V and VI) divisions and each was analyzed separately. Systematic random sampling was performed by randomly translating a 500X0500 µm² grid onto the section of interest. A 30 X 30 X 19 µm³ optical dissector was created at each sampling site using a 63X objective (oil; numerical aperture, 1.3). Only the profiles falling within the counting frame that did not contact the exclusion lines and that came into focus within the predetermined 19 µm-thick optical dissector positioned 2.5 µm below the surface of the mounted section were counted. The software package converted the counts into total numbers of NeuN-positive and negative cells. We counted cells in the caudate putamen and frontal cortex of only one hemisphere. The data were multiplied by 2 to obtain estimates of cell numbers for both the hemispheres.

To determine if NeuN-positive cells were distributed throughout the optical dissector (as opposed to being clustered at either surface), we calculated the percentage of NeuN-positive cells (as a percentage of total NeuN-positive cells) located throughout the entire thickness of the optical dissector for a given section. We used 4 sections from each of the 3 experimental groups and average values for each group were calculated (Fig 4D).

Statistical analyses

Overall differences among the L-DOPA, ASC and WATER groups were analyzed for statistical significance by one-way analysis of variance (ANOVA). If significant differences were found then we compared the mean values between each pair of groups using Tukey's Multiple Comparisons test to determine more specifically the source of the differences. The statistical analyses were performed using the Prism 4 for Macintosh statistical analysis package (GraphPad Software Inc, San Diego, CA).

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Figure 1.

A schematic representation of the experimental design. L-DOPA + Ascorbic Acid (L-DOPA), Ascorbic acid alone (ASC) or plain drinking water (WATER) was administered to pregnant mice from the 11th day of pregnancy until parturition. On embryonic day 13 (E13) or E15, a single injection of bromodeoxyuridine (BrdU) was administered to the pregnant mice. Four types of analyses were performed on fetal and postnatal brains to assess the effects of the experimental treatments. (A) Cell proliferation in the forebrain neuroepithelial domains was analyzed on E13 or E15 by calculation of 2.0 hr BrdU labeling index (LI). (B) BrdU LI was analyzed and BrdU-NeuN double labeling was performed in different regions of the forebrain of the offspring on postnatal day 21 (P21). (C) Cell death was analyzed in the embryonic and postnatal brains by using TUNEL histochemistry. (D) stereological methods were used to estimate total numbers of NeuN-positive and NeuN-negative cells in the P21 caudate putamen and frontal cortex.

Figure 2.

Micrographs of histological sections processed for bromodeoxyuridine (BrdU), NeuN, tyrosine hydroxylase (TH) and TUNEL labeling. BrdU and NeuN double-labeling in layer II of the frontal cortex of a P21 brain from the WATER group is shown at low (A) and higher (B) magnifications. NeuN (red) and BrdU (green) double-labeled nuclei (yellow; white arrows in A and B) appear yellow. Virtually all (~95%) of the BrdU-labeled nuclei were also NeuN-labeled. Indeed, in the micrographs in A and B there is no BrdU-only labeled nucleus. TH immunohistochemistry was performed on sections from the rostral (C) and caudal (D) forebrain of an embryonic day 15 mouse. TH-labeled fibers (green) appear restricted to postmitotic regions of the basal forebrain (white star in C and D) such that neuroepithelial domains of the

lateral, medial and caudal ganglionic eminences (LGE, MGE and CGE, respectively) appear devoid of TH-labeled profiles at this magnification. However, at higher magnification (E and F), in the brain of an embryonic day 15 mouse that was exposed to BrdU for 6.5 hr prior to sacrifice, TH-positive profiles (red; white arrows in E and F) are in close proximity to BrdU-positive proliferating neuroepithelial cells (green) in the MGE (E) and CGE (F). An example of TUNEL histochemistry on sections of postnatal day-7 mouse brain followed by propidium iodide labeling (G and H). TUNEL positive profiles are green and propidium iodide labeled nuclei are red (G and H). Lower power micrograph (G) shows TUNEL positive profiles (white arrowhead) in the subventricular zone (white star) as well as in the caudate-putamen (CP; white arrows). Higher power micrograph (H) taken from the caudate-putamen shows TUNEL-positive profiles (white arrows) that are also labeled with propidium iodide. The scale bar in D applies also to C and that in F also to E.

Figure 3.

Analysis of bromodeoxyuridine (BrdU) labeling index (LI) in the different regions of the forebrain of postnatal day 21 mice that were exposed to a single BrdU injection on embryonic day 15. A and B are low magnification views of two paraffin-embedded sections of the brain in the coronal plane showing the sites of analysis of BrdU LI in the frontal cortex (FC), the dorso-medial (DM) and ventro-lateral (VL) subdivisions of the caudate-putamen (CP), the nucleus accumbens (NAc) and the globus pallidus (GP). A microscope field within the boxed area labeled FC in A is shown in C. Cellular layers (layer II–VI) of the gray matter of the FC were divided into 20 bins (C). Each bin represented 5% of the radial thickness between layers II and VI. That is, the thickness from layer II to VI was considered to be 100%. BrdU-labeled and non-BrdU-unlabeled (basic fuchsin only labeled) nuclei were counted and BrdU LI was calculated for each bin. As shown in the charts in C, the BrdU LI was higher in the superficial 25% of the gray matter, which was determined by histological examination of the sections to correspond approximately to layers II and III. The BrdU LI in the superficial 25% of the gray matter was significantly decreased in the L-DOPA group compared to the ASC and WATER groups. The average numbers of all cells (BrdU-labeled plus non-BrdU-labeled) did not show significant variation across the different layers or different groups.

Figure 4.

An unbiased stereological technique, the optical fractionator was used to estimate the total number of NeuN-positive and NeuN-negative cells in the caudate-putamen and frontal cortex. The boundaries of the caudate putamen and frontal cortex were drawn at low magnification (A). In rostral sections, the ventral striatum was excluded from analysis by a line drawn from the ventral tip of the lateral ventricle (LV) to the dorsal border of the piriform cortex (A). Frontal agranular cortex was identified by the absence of layer IV. Since distinguishing between primary and secondary motor areas was difficult, the two areas collectively are called frontal cortex. Cell numbers were separately estimated for layers II–III and V–VI. Higher power images from a histological section processed for NeuN immunohistochemistry using diaminobenzidine as chromagen and stained with cresyl violet. NeuN-positive (brown) and NeuN-negative (blue) cells are shown from the frontal cortex (B) and caudate putamen (C). To confirm that NeuN-positive cells were distributed throughout the thickness of the optical

dissector, percentage of NeuN-positive cells at 10 percentile increments through the dissector was analyzed for each experimental group (D). WM = white matter; AC = anterior commissure.

Table 1

Mean±SEM values of BrdU LI in the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE), caudal ganglionic eminence (CGE) and neuroepithelium of the frontal cortex (FC) in the three experimental groups (L-DOPA, ASC and WATER) at E15. The data were analyzed for the entire neuroepithelium, combining the ventricular zone (VZ) and subventricular zone (SVZ), as well as for VZ and SVZ separately. ANOVA was performed to determine if the overall group differences were statistically significant ($p<0.05$). When ANOVA revealed significant differences in a particular parameter, Tukey's Multiple Comparison Test was performed to determine the source (i.e. the experimental treatment) of the difference.

	L-DOPA	ASC	Water
E13			
LGE	0.34±0.02	0.31±0.02	0.36±0.03
MGE	0.36±0.03	0.38±0.03	0.38±0.04
CGE	0.26±0.02	0.30±0.02	0.28±0.02
FC	0.30±0.03	0.25±0.03	0.28±0.05
E15			
LGE ^{*†}	0.20±0.01	0.28±0.02	0.30±0.03
MGE	0.28±0.02	0.27±0.02	0.26±0.01
CGE	0.27±0.02	0.25±0.01	0.25±0.02
FC [*]	0.18±0.02	0.25±0.02	0.26±0.01

Significant differences ($p<0.05$) revealed by the Tukey's test are indicated by the following symbols:

* = significant difference between L-DOPA and Water;

† = significant difference between L-DOPA and ASC

Table 2

Mean±SEM values of BrdU LI in the E15 lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE), caudal ganglionic eminence (CGE) and frontal cortical neuroepithelium (FC) following administration of the D1-receptor agonist SKF 81297 or saline (control). ANOVA did not reveal significant differences between groups for the MGE or CGE ($p<0.05$). The data for LGE and FC are taken from our earlier publication [43].

	SKF 81297		Saline
	10 mg/kg	20 mg/kg	
MGE	0.28±0.03	0.26±0.03	0.26±0.01
CGE	0.22±0.02	0.24±0.01	0.25±0.02
LGE	0.21±0.01*	N/A	0.30±0.02
FC	0.23±0.03	0.20±0.01*	0.26±0.01

* = BrdU LI was significantly lower in the SKF 81297 group compared to saline group ($p<0.05$).

N/A: Since 10 mg/kg SKF 81297 showed significant effects in the LGE, 20 mg/kg dose was not tested (from Popolo et al., 2004).

Table 3

Mean+SEM values for BrdU LI and numerical density of all cells (BrdU labeled and unlabeled nuclei/1,000 μm^2) in the dorso-medial and ventro-lateral divisions of the caudate-putamen of P21 mice in the three experimental groups (L-DOPA, ASC and WATER). ANOVA was performed to determine if the overall group differences were statistically significant ($p<0.05$). Since ANOVA revealed significant differences in all measurements, Tukey's Multiple Comparison Test was performed to determine the source of the difference.

	L-DOPA	ASC	WATER
	BrdU LI		
Dorso-medial ^{†§}	0.10±0.01	0.20±0.02	0.14±0.02
Ventro-lateral ^{†§}	0.08±0.01	0.23±0.02	0.11±0.01
	All nuclei		
Dorso-medial	2.98±0.18	2.76±0.16	2.51±0.15
Ventro-lateral	2.73±0.04	2.96±0.08	2.52±0.23

Significant differences ($p<0.05$) revealed by the Tukey's test are indicated by the following symbols:

[†] = significant difference between L-DOPA and ASC;

[§] = significant difference between ASC and Water.

Table 4

Mean+SEM values for BrdU LI and numerical density of all cells (BrdU labeled and unlabeled nuclei/1,000 μm^2) in the nucleus accumbens of P21 mice in the three experimental groups (L-DOPA, ASC and WATER). ANOVA was performed to determine if the overall group differences were statistically significant ($p<0.05$). Since ANOVA revealed significant differences, Tukey's Multiple Comparison Test was performed to determine the source of the difference. In this instance, significant differences ($p<0.05$) were revealed by the Tukey's test only between L-DOPA and ASC groups ($\dagger$).

	L-DOPA	ASC	WATER
BrdU LI $\dagger$	0.06 $\pm$ 0.02	0.16 $\pm$ 0.02	0.11 $\pm$ 0.02
All Nuclei	2.0 $\pm$ 0.13	1.68 $\pm$ 0.29	2.07 $\pm$ 0.15

Mean+SEM values of BrdU LI and numerical density of all cells (BrdU labeled and unlabeled/1,440µm²) in the different layers of the frontal cortex of P21 mice in the three experimental groups (L-DOPA, ASC and WATER). ANOVA was performed to determine if the overall group differences were statistically significant ($p<0.05$). Since ANOVA revealed significant differences in BrdU LI in layers II–III and layers II–VI, Tukey’s Multiple Comparison Test was performed on those measurements, which revealed significant differences ($p<0.05$) between L-DOPA and ASC (†).

Table 5

	Layers II–III (upper 25%)		Layers V–VI (lower 75%)		Layers II–VI (100%)	
	BrdU LI †	All nuclei	BrdU LI	All nuclei	BrdU LI †	All nuclei
L-DOPA	0.10±0.00	1.28±0.05	0.02±0.01	1.27±0.12	0.03±0.01	1.42±0.14
ASC	0.18±0.03	1.47±0.07	0.02±0.01	1.22±0.04	0.07±0.01	1.45±0.04
WATER	0.13±0.02	1.26±0.13	0.01±0.00	1.34±0.08	0.05±0.01	1.47±0.05

Table 6

Mean $\pm$ SEM values of percentage of BrdU-labeled nuclei that are also NeuN-labeled in 1000 μ m² sector of the dorso-medial and ventro-lateral divisions of the caudate putamen and in layers II–III of the frontal cortex in the three experimental groups (L-DOPA, ASC and WATER) at P21. ANOVA was performed to determine if the overall group differences were statistically significant ($p<0.05$). Since ANOVA did not reveal significant differences, Tukey's Multiple Comparison Test was not performed.

	Caudate-putamen		Frontal Cortex
	Dorso-medial	Ventro-lateral	
L-DOPA	94.47 $\pm$ 0.98	91.67 $\pm$ 8.33	95.83 $\pm$ 4.17
ASC	95.15 $\pm$ 4.85	95.00 $\pm$ 5.00	97.12 $\pm$ 2.88
WATER	98.84 $\pm$ 0.09	97.62 $\pm$ 2.38	93.74 $\pm$ 0.29

Table 7

Mean±SEM values of total numbers of NeuN-positive, NeuN-negative and all cells in the P21 caudate-putamen and frontal cortex estimated by stereology. Cell numbers were estimated separately for the upper (II–III) and lower (V–VI) layers of the frontal agranular cortex. ANOVA showed that the differences among the 3 groups were not statistically significant ($p>0.05$). Since ANOVA did not reveal significant differences, Tukey's Multiple Comparison Test was not performed.

	Caudate-Putamen (10^5)		
	L-DOPA	ASC	WATER
NeuN-positive	8.0±0.4	7.7±0.5	8.1±0.2
NeuN-negative	7.7±0.2	6.4±0.3	7.0±0.4
Total cells	16.0±0.5	14.0±0.7	15.0±0.4
	Frontal Cortex All layers (10^5)		
	L-DOPA	ASC	WATER
NeuN-positive	2.5 ± 0.1	2.6 ± 0.2	2.9 ± 0.2
NeuN-negative	2.3 ± 0.1	2.2 ± 0.1	2.6 ± 0.1
Total cells	4.9 ± 0.2	4.9 ± 0.2	5.5 ± 0.3
	Frontal Cortex Layers II–III (10^5)		
	L-DOPA	ASC	WATER
NeuN-positive	1.5 ± 0.7	1.5 ± 0.1	1.7 ± 0.1
NeuN-negative	1.3 ± 0.6	1.3 ± 0.1	1.4 ± 0.1
Total cells	2.8 ± 0.1	2.8 ± 0.2	3.1 ± 0.2
	Frontal Cortex Layers V–VI (10^5)		
	L-DOPA	ASC	WATER
NeuN-positive	1.1 ± 0.1	1.1 ± 0.1	1.3 ± 0.1
NeuN-negative	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.1
Total cells	2.1 ± 0.1	2.1 ± 0.1	2.5 ± 0.2

Table 8

Mean±SEM values of percent TUNEL labeling (% TUNEL-positive profiles ÷ propidium iodide-positive profiles) per microscope field in the lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE) and caudal ganglionic eminence (CGE) at E15 and frontal cortex (FC) and caudate-putamen (CP) at P0, P7 and P14 in the 3 experimental groups (L-DOPA, ASC and WATER). ANOVA was performed to determine if the overall group differences were statistically significant ($p<0.05$). Since ANOVA did not reveal significant differences, Tukey's Multiple Comparison Test was not performed.

	L-DOPA	ASC	WATER
E15			
LGE	0.00±0.00	0.09±0.05	0.07±0.03
MGE	0.00±0.00	0.04±0.04	0.08±0.08
CGE	0.00±0.00	0.04±0.04	0.11±0.08
FC	0.07±0.05	0.04±0.04	0.03±0.03
P0			
CP	1.22±0.45	0.95±0.32	1.06±0.30
FC	0.18±0.18	0.08±0.08	0.08±0.08
P7			
CP	0.23±0.13	0.45±0.22	0.55±0.28
FC	0.0±0.0	0.10±0.05	0.19±0.10
P14			
CP	0.40±0.13	0.53±0.06	0.41±0.10
FC	0.76±0.43	0.53±0.08	0.66±0.08