

Neonatal 5,7-DHT Lesions Cause Sex-Specific Changes in Mouse Cortical Morphogenesis

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SUMMARY

Both monoaminergic and cholinergic afferent projections to the neocortex putatively modulate cortical morphogenesis and plasticity. Previously we showed that neonatal electrolytic lesions of the cholinergic nucleus basalis magnocellularis (nBM) projections to the neocortex result in significant decreases of cortical layer width that correlate with cognitive alterations. Such electrolytic lesions, performed for lack of a selective neurotoxin in mice, may affect mono- aminergic fibers of passage. Here, we investigate the effects of neonatal 5,7 dihydroxytryptamine (5,7-DHT) focal injections into the nBM region on cortical laminar morphology in adult male and female mice. 5,7-DHT lesions on the first postnatal day resulted in significant cortical depletion of both serotonin and norepinephrine that attenuated with age. Generally, cortical layer widths increased in response to the lesion; the effects were layer, region, and sex specific. Previous reports from our laboratories described long-term behavioral alterations after comparable focal, neonatal 5,7-DHT lesions. The studies described here provide an anatomical basis for such behavioral alterations. Our data suggest that monoaminergic and cholinergic projections

to the cortex may have opposite effects on the developing cortical neuropil. Jointly, our morphological and behavioral findings may have important implications for a variety of developmental disorders in humans and provide some insights into sex differences in the penetrance of these disorders.

INTRODUCTION

The development of the mammalian cerebral cortex involves a precisely timed series of events to create its characteristic cytoarchitecture and neural circuitry. Once matured, this circuitry is critical for the appropriate integration of sensory/motor performance, as well as cognitive functions. A review of the literature on developmental disorders attests that even subtle disruptions of cortical architecture and connectivity can result in pronounced behavioral alterations that persist into adulthood (Berger-Sweeney & Hohmann, 1997).

A wealth of data indicates that monoaminergic and cholinergic modulatory projections can influence cell proliferation, morphogenesis, and plasticity in the central nervous system (Azmitia, 1999; Berger-Sweeney & Hohmann, 1997; Foehring & Lorenzon, 1999; Hohmann & Berger-Sweeney, 1998a; Lauder, 1993; Levitt et al., 1997; Mattson, 1988). Noradrenergic neurons in the locus coeruleus, serotonergic neurons primarily in the dorsal and median raphe, and cholinergic neurons in the nucleus basalis magnocellularis are generated in

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the second and third gestational weeks in rodents. Axons from these neurons begin to project to upstream targets via the medial forebrain bundle and internal capsule, respectively, during late gestation and on the first few weeks postnatally (for review see Berger-Sweeney & Hohmann, 1997). As they reach the cortex, these afferents can influence the development of their target structures. Numerous studies show that altering monoaminergic and cholinergic transmitters during the late prenatal and early postnatal periods produce a variety of abnormalities in the developing neocortex. Behavioral consequences have been identified in response to neonatal alterations of each of these afferent transmitter systems (Berger-Sweeney & Hohmann, 1997; Hohmann & Berger-Sweeney, 1998a; Kolb & Wishaw, 1998).

The specific effects of neonatal noradrenergic (NE) depletion on cortical morphogenesis are somewhat contradictory, and their interpretation is compounded by substantial differences in lesion approaches (see Berger-Sweeney & Hohmann, 1997). The morphogenetic changes that are observed most consistently with neonatal NE depletions are reductions in dendritic branching and spine densities, as well as overall reductions in brain size (Brenner et al., 1985; Felten et al., 1982; Kolb & Sutherland, 1992; Loeb et al., 1987; Onteniente et al., 1980; Osterheld-Haas & Hornung, 1996). On the other hand, enhancement of central noradrenergic transmission neonatally appears to increase the cortical neuropil (Ruiz et al., 1997).

Recent studies employing different methods for depleting serotonin (5-HT) have demonstrated consistent, albeit subtle, effects on cortical maturation. Disruption of normal serotonergic projections within the first few postnatal days results in delayed thalamic fiber innervation and reduced size of cytoarchitectonic 'barrels' in the barrel field area (Woolsey & Van der Loos, 1970) of rat sensory cortex (Blue et al., 1991; Bennett-Clarke et al., 1994; Rhoades et al., 1998; Turlejski

et al., 1997; Vitalis et al., 1998). In addition, afferents from specific sensory relay nuclei in the thalamus transiently express high affinity serotonin uptake pumps, as well as 5-HT 1B receptors, and store serotonin during the critical period of barrel field development (Bennett-Clarke et al., 1993; Bruning & Liagos, 1997; Hansson et al., 1998; Lebrand et al., 1998; Mansour-Robaey et al., 1998). Moreover, in culture, neurons of thalamic origin can modulate their process outgrowth in response to serotonin (Lieske et al., 1999). Jointly, these observations suggest an interaction between serotonergic and thalamocortical (glutamatergic) afferents which, in turn, impacts cortical morphogenesis. 5-HT appears to exert direct effects on neuropil maturation in the neocortex and hippocampus as well (Mazur et al., 1997; Osterheld-Haas & Hornung, 1996; Yan et al., 1997). Interestingly, too much 5-HT may be as detrimental to cortical development as is too little. Monoamine oxidase inactivation, either genetically or pharmacologically, leads to significant increases of cortical serotonin and precipitates a complete absence of cortical barrels in mouse (Vitalis et al., 1998). A similar U-shaped, dose-response curve has been reported for the morphogenesis of other brain regions after manipulations of serotonergic inputs (Mooney et al., 1998).

The cortical effects of 5-HT and NE during development have assumed increasing clinical relevance. Imbalances in serotonergic and noradrenergic neurotransmission have been implicated in a variety of neurological and psychiatric disorders of developmental onset, such as autism, schizophrenia, and attention deficit hyperactivity disorder (ADHD) (Anderson et al., 1990; Chugani et al., 1999; Cook & Leventhal, 1996; Glenthøj & Hemmingsen, 1999; Hanna et al., 1996). Skewed sex ratios have been observed in many such disorders. Moreover, serotonergic metabolism responds to perinatal stressors in a highly sex-specific way (Alonso et al., 1991; Flemming et al., 1986; McGrath et al., 1997; Resnicov & Nosenko, 1996; Steward, 1991).

We have shown in a recent series of papers (Arters et al., 1998; Hohmann & Berger-Sweeney, 1998b) that lesions of basal forebrain cholinergic (nBM) afferents to the somato-sensory cortex in the mouse result in sex-specific alterations in neo-cortical morphogenesis that are associated with impairments in cognitive behaviors. Specifically, nBM lesions significantly reduce cortical layer width in the barrel field and in the posterior somato-sensory cortex. These effects significantly correlate with cognitive deficits in male, but not in female, mice. Because these nBM lesions are made electrolytically, for lack of an effective cholinergic neurotoxin in mouse, we were concerned that medial forebrain bundle monoaminergic fibers may be inadvertently damaged. As such, cholinergic depletions may be compounded by serotonin and/or norepinephrine depletions in the developing cortex in that lesion model. Therefore, here we investigated the effects of 5,7-DHT injections directly into the basal forebrain, using the same coordinates and the same developmental age as those described for neonatal nBM lesions (Hohmann et al., 1988a). In a previous paper, we characterized the effects of such neonatal, focal 5,7-DHT injections on cognitive behaviors in adult mice of both sexes (Berger-Sweeney et al., 1998). Mice with neonatal monoamine depletions displayed retention deficits on passive avoidance and improved performance on delayed-non-match-to-sample odor discriminator task in adulthood.

The present study investigates the morphological effects of both bi- and unilateral 5,7-DHT injections in male and female BALB/cByJ mice in three functionally different cortical regions: the barrel field region, as well as the somato-motor cortex regions anterior and posterior to it. In a previous study, we observed hemisphere and sex-selective effects after unilateral nBM electrolytic lesions (Hohmann et al., 1999). Therefore, here we aim to assess whether similar changes are precipitated with neonatal 5,7-DHT injections.

MATERIALS AND METHODS

All animals were derived using our own BALB/c ByJ breeding colony at Morgan State University. Lesion and control animals were litter mates derived from the same mothers. All animals evaluated in this study were subjected to hypothermia anesthesia.

Lesion Method

As previously described (Berger-Sweeney et al., 1998), mice were removed from their mothers 12 to 24 h after birth. The pups to be lesioned were immobilized in a specially designed Plexiglas-mold head holder mounted to a David Kopf stereotaxic apparatus (see (Hohmann et al., 1988a; Hohmann & Berger-Sweeney, 1998b)). A fine cannula (28 gauge) was inserted through the skull 1 mm lateral to the midline and 1.5 mm anterior to the fronto-nasal suture at a vertical angle of 49° and a horizontal angle of 5° and lowered into the brain. The monoaminergic toxin 5,7 dihydroxytryptamine (5,7-DHT) (5 mg/ml, 0.5 µl/total injection/animal) was injected at depths of 3.0, 3.5, and 4.0 mm into the nBM area of the right hemisphere or bilaterally; control animals received equal volumes of the carrier (H₂O) or were submitted to hypothermia anesthesia only. All control litter mates to the bilaterally 5,7-DHT injected mice received bilateral carrier injections. In contrast, the litter mates of unilaterally lesioned mice received hypothermia anesthesia only. The lesioned and control pups were transferred to a heating pad for 30 min to regain normal body temperature. Afterward, the pups were returned to their mothers until the time of sacrifice for neurochemistry or histology.

Neurochemistry

Brains for neurochemistry were taken on post-natal days 7–9 (PND 7–9), at PND14 and at young

adulthood (3 to 5 mo). The dorsal cortex was dissected quickly on ice, weighed, frozen on dry ice, and then stored at -70°C . Monoamine levels were ascertained by HPLC analysis with electrochemical detection, according to the methods of Zaczek et al. (Zaczek and Coyle, 1982) as described previously (Berger-Sweeney et al., 1998; Hohmann et al., 1988b).

Histology

Brains for histology were taken at 3 to 5 mo postnatally. The mice were perfused first with 0.9% saline followed by 0.1M PO_4 buffer (pH 7.4) containing 10% Formalin. Afterward, the brains were removed and submerged in 20% sucrose phosphate-buffered formalin and stored for 4 to 5 h in the refrigerator. After 4 to 5 h, the brains were transferred into a vial containing 20% sucrose in 0.1M phosphate buffer and stored for another 12 to 20 h in the refrigerator before freezing them quickly in isopentane at -25°C . The brains were sectioned at 50 μm on a frozen stage microtome (Microm, Zeiss), and alternate sections were processed for Nissl staining or acetylcholinesterase (AChE). AChE and Nissl staining were performed as previously reported (Hohmann & Ebner, 1985; Hohmann et al., 1988a; Hohmann & Berger-Sweeney, 1998b). The histology of each control and lesioned animal was subjected to careful qualitative examination. Animals with noticeable tissue damage connected to the injection site were eliminated from further study.

MCID

Morphometric analysis was conducted using a computerized image analysis system (MCID M4 Image Analysis software and a Dell Pentium computer). As recently described (Hohmann & Berger-Sweeney, 1998b), analyses were performed for three functionally different regions of the dorsal

cortex: (1) Area A, comprising the region anterior to the barrel fields of the face representation, (2) area B, including the postero-medial barrel subfield (PMBS) representation of the mystacial vibrissae (Woolsey & Van der Loos, 1970), and (3) area C, comprising the sensory cortex posterior to the barrel fields. Images of the tissue sections were captured via a Hamamatsu CCD (with C24 control box) video camera displayed on a high resolution videoscreen and digitized. As illustrated in Fig. 1, the cytoarchitectonic boundaries of individual cortical layers were clearly discernable, with the exception of boundaries between layers II and III. Four separate measurements of total cortex and layers VI, V, IV, and II+III jointly were performed within each hemisphere of each section, as shown in Fig. 1. Statistical analyses were performed using factorial analysis of variance (ANOVA) (StatView, Abacus Concepts).

RESULTS

Mice of both sexes, receiving bilateral or unilateral (right hemisphere) injections of 5,7-DHT at birth, were examined for qualitative and quantitative changes in cortical cytoarchitecture in adulthood. All quantitative assessments of cortical layer width were performed on Nissl-stained sections. The adjacent AChE-stained sections were used to assess the possible effects of the lesion on cholinergic afferents to the cortex. Previous studies showed AChE in mouse cortex to be specifically associated with cholinergic afferents (Hohmann & Ebner, 1985; Hohmann et al., 1988a). Bilateral lesion assessments were made to allow morphological comparisons with bilaterally neonatally nBM lesioned mice and to evaluate possible morphological correlates of the bilaterally 5,7-DHT lesioned mice tested in our prior behavioral study (Berger-Sweeney et al., 1998). On the other hand,



Fig. 1: Digitized image of a coronal section through the somato-sensory cortex of an uninjected control mouse. Note that boundaries between cortical layers are clearly discernable. The white lines show the computer overlay of measurements of individual cortical layers and total layer width as performed on the MCID system.

the quantitative assessment of unilateral lesion effects in adulthood allowed us to compare cortical responses ipsi- and contralateral to the neonatal 5,7-DHT injection within the context of potential hemispheric asymmetries in control animals. An additional group of unilaterally 5,7-DHT lesioned mice was prepared for neuro-chemical assessment of the lesion efficacy at 1 and 2 wk postnatally and in adulthood.

Neurochemistry

Monoamine content was assessed in 5 uninjected control and 5 unilaterally nBM lesioned mice at 1 wk post-lesion and in 3 uninjected control and six lesioned mice 2 wk post-lesion. Because of the small numbers of animals and the

small volume of tissue, male and female mice were pooled for these assessments. Ipsilateral to the lesion, serotonin (5-HT) was significantly decreased by 63.3% ($P=0.004$) at 1 wk and by 85.5% ($P=0.002$) at 2 wk compared with both contralateral hemisphere and control values. The NE depletions were smaller: NE was significantly decreased by 33.3% at 1 wk ($P=0.02$) and by 19.3% at 2 wk post-lesion, which was no longer statistically significant. No significant differences between contralateral to lesions (left) hemisphere samples and control samples were apparent for either NE or 5-HT. Also, significant right/left neurochemical asymmetries were not apparent for NE or 5-HT levels in control brains.

By adulthood, depletion of both 5-HT and NE had attenuated considerably relative to the perinatal assessments. The 5-HT levels were significantly

decreased by 29.7% ($P=0.02$) in males compared with the contralateral hemisphere and by 40.4% ($P=0.02$) in females. NE decreases in the ipsi-lateral to lesion hemisphere were no longer significantly different from those in the contra-lateral hemisphere and measured 19.06% in males and 16.43% females. The male/female differences were not statistically significant in these adult animals, but the numbers of brains assessed may have been too small to detect such subtle sex differences.

Histology

Mice included in the morphometric analyses (lesioned at birth and analyzed at 3 to 5 mo postnatally) had very small but usually identifiable cannula tracks near the site of 5,7-DHT or carrier injection. Animals showing histologic damage to brain structures surrounding the injection site were eliminated from quantitative analysis. Qualitative assessment of the cerebral cortex in Nissl-stained sections revealed no obvious alteration of cortical cytoarchitecture; likewise, changes in AChE patterns in the cortex were not found. On the other hand, quantitative comparisons of lesioned and control mice revealed significant differences in the thickness of a variety of cortical layers. In most instances, the lesion induced an increase in cortical thickness. Cortical layer width changes often followed a sexually dimorphic pattern. For the cohort of unilaterally lesioned animals, pervasive asymmetries between the right and left hemispheres were apparent in control mice. In many instances, the asymmetries were greatly attenuated after the 5,7-DHT lesion; although in a few cases, male/female differences in hemispheric asymmetry were exacerbated following the lesion. Lesion-induced alterations in cortical thickness varied with the cortical areas analyzed.

The effects of **bilateral** 5,7-DHT injections into the neonatal basal forebrain were assessed in nine carrier injected control males, six carrier

injected control females, seven lesioned females, and eight lesioned males. Analyses were made first with males and females combined and second with each sex separately. In both instances, the right and left hemispheres were measured jointly. This strategy allowed us to examine the overall and sex-specific lesion-induced effects. Morphometric computerized analysis was conducted separately on Nissl-stained sections for anterior, barrel, and posterior cortices. A summary of the findings for these mice is shown in Table 2A.

In the **anterior cortex**, as shown in Fig. 2A, bilateral lesions resulted overall in significant ($P<0.0001$) increases in total cortical width relative to controls when both sexes were assessed jointly. When assessed separately by sex, only males showed significant width increases relative to controls ($P=0.001$); female differences approached significance ($P=0.07$), however. Similarly, layers II/III were increased significantly in both sexes measured jointly ($P=0.001$), which was due predominantly to a significant ($P=0.01$) increase in males as well (see Fig. 3A). The same trend of greater lesion-induced increases in width in male vs. female mice continued in layers IV and V. In both instances, there were significant ($P=0.003$) increases in laminar widths for both sexes measured jointly. As shown in Fig. 4A, in layer VI both males ($P=0.0004$) and females ($P=0.05$) displayed significant increases in widths compared with controls, although once more the differences were more pronounced in males.

In the **barrel cortex**, the overall (male and female) total cortical width was significantly increased in lesioned vs. control mice, as illustrated in Fig. 2B. In this cortical region, however, female width increases were somewhat more pronounced ($P=0.001$) than those of males ($P=0.03$). Likewise, in layers II/III (see Figure 3B), the overall (male plus female) significant differences ($P=0.001$) were due to highly significant differences in females ($P=0.0005$) in the absence of significant

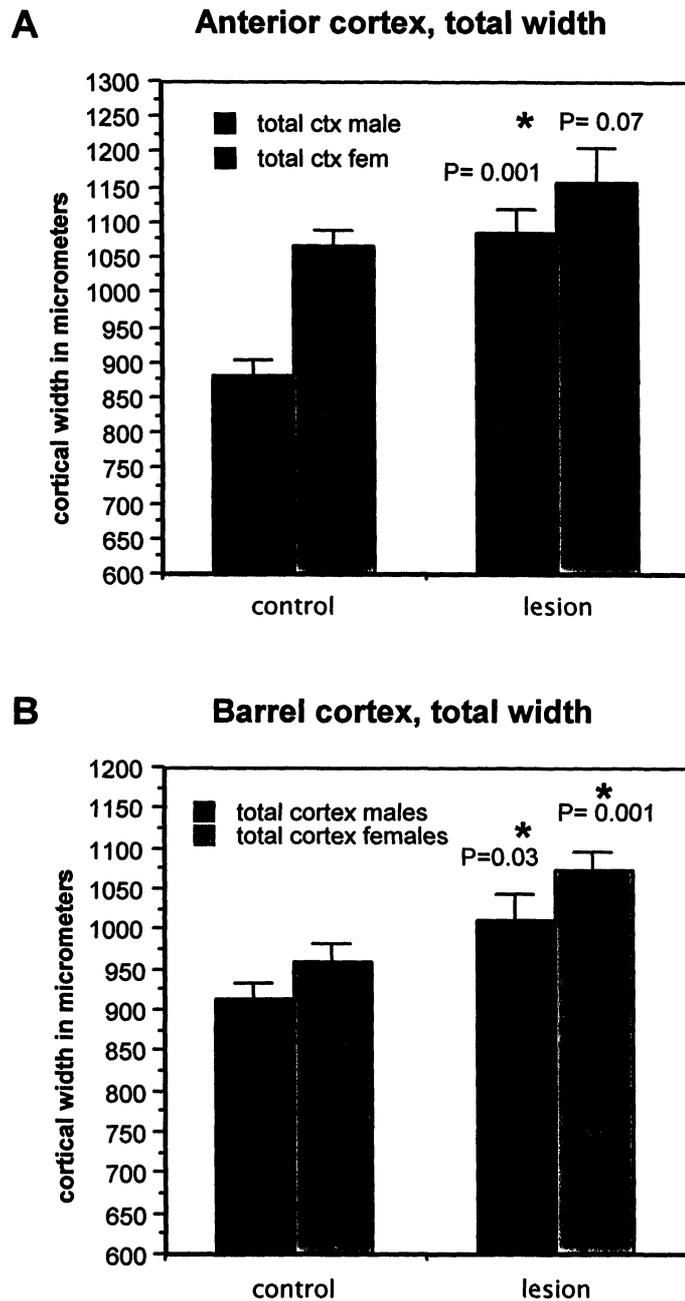


Fig. 2: Comparison of significant changes in total cortical width in anterior (a) and barrel field cortex (b) following bilateral neonatal 5,7-DHT injections; * denotes significant lesion related differences. In anterior cortex, only males displayed significant increases in cortical width, with females nevertheless displaying a strong trend in the same direction. In contrast, in barrel cortex, both males and females show significant differences that are more robust in the female in this region. Note the considerable sex difference in cortical width in control animals (significant in anterior cortex, see text) and their attenuation following the lesion in anterior cortex.

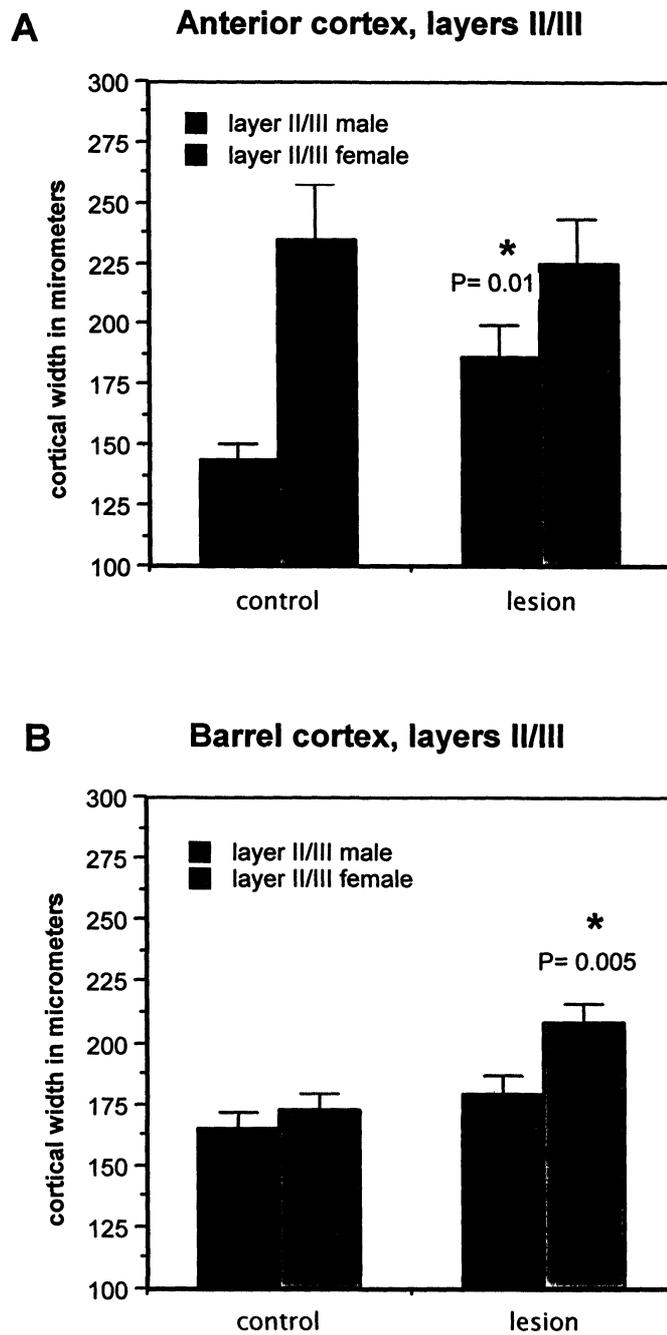


Fig. 3: Comparison of significant changes in layers II/III in anterior (a) and barrel (b) cortex following bilateral neonatal lesions; * denotes significant lesion related differences. Note that males are significantly affected in anterior cortex whereas females are significantly affected in barrel cortex. In total cortical widths in anterior cortex, significant sex differences (see text) in controls are attenuated by the lesion. In contrast, sex differences are exacerbated in barrel cortex.

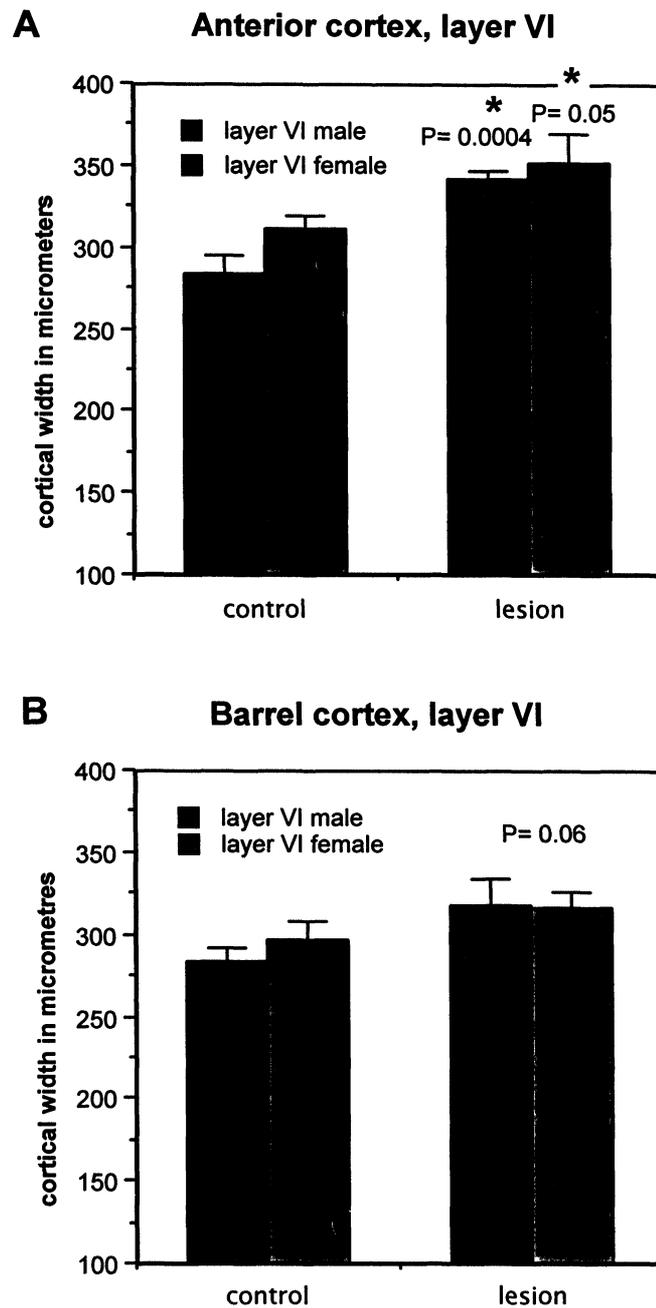


Fig. 4: Comparison of significant changes in layer VI in anterior (a) and barrel (b) cortex following bilateral lesions; * denotes significant lesion related differences. As before, males are more prominently affected in anterior cortex and sex differences are attenuated by the lesion.

differences in males. Cortical layers IV and V showed overall significant increases in width in lesioned vs. control mice ($P=0.02$); each sex alone, however, did not reach significance. In layer VI there was a trend toward increased cortical widths in lesioned mice ($P=0.06$).

For the **posterior cortex**, the lesioned mice displayed significant increases in the width of the total cortex ($P=0.005$), as well as layers II/III ($P=0.03$) and IV ($P=0.04$) when males and females were assessed together. Each sex assessed alone, however, did not reach significance. The significant increase in overall (male plus female) layer V width ($P=0.0001$) was predominantly due to a significant ($P=0.003$) increase in female layer width. No differences in layer width were apparent in layer VI for this area.

For the assessment of **unilateral** (right hemisphere) lesions, we analyzed 9 normal uninjected adult male and 11 normal uninjected adult females, as well as 12 neonatally 5,7 DHT lesioned males and 10 females. The data are detailed in Table 1; a graphic summary of the data is given in Table 2B.

In the **anterior cortex**, no significant lesion effects were measurable in any cortical layer. In control mice, significant sex differences ($P=0.02$ – 0.0001) were pervasive, although not always apparent in both hemispheres (see Table 1). In many instances, significant hemispheric asymmetries were measurable ($P=0.0002$ for layer VI and $P=0.005$ for layer IV) and differed significantly by sex ($P=0.0008$ for layer IV). We observed that hemispheric asymmetries and sex differences were often attenuated after the lesion; hence, significant sex \times lesion status interactions could be seen in layer VI ($P=0.03$) and layers II/III ($P=0.02$) (see Table 1).

In the **barrel cortex**, significant lesion-induced overall (male plus female) increases in width were apparent in the ipsilateral hemisphere for the total cortex ($P=0.04$), for layer IV ($P=0.03$), and for

layers II/III ($P=0.49$) (see Table 1). In layer IV, this significant increase was due predominantly to increases in the female, whereas layers II/III and total cortical width showed increases in lesioned mice of both sexes (Table 1). No significant lesion effects emerged in layers V and VI; although a trend ($P=0.09$) was observable in layer V when both sexes were assessed together. Significant sex differences among controls were apparent in layers II/III ($P=0.0001$), VI ($P=0.02$), and in the total cortex ($P=0.0001$) in both hemispheres; yet pronounced hemisphere asymmetries were apparent in the same places (total cortex [$P=0.0001$], layer VI [$P=0.0001$], layers II/III [$P=0.05$]). As observed in the anterior cortex, lesions interacted with these sex and hemispheric differences by attenuating the hemisphere asymmetries. This interaction is reflected in significant sex \times hemisphere \times lesion changes for layer IV ($P=0.05$).

In the **posterior cortex**, significant lesion-induced width increases were seen for the total cortical width ($P=0.042$) as well as in layers II/III ($P=0.038$) and IV ($P=0.023$), when both sexes were measured jointly. Whereas increases in the total cortical width, ipsilateral to the lesion (right hemisphere), reflected significant increases in both sexes, the increases in layers II/III and IV were due entirely to a significant increase in females and occurred bilaterally (see Table 1). In contrast to the anterior and barrel cortex, layer V in the posterior cortex showed a small but significant ($P<0.05$) decrease in females bilaterally (see Table 1). Similarly to the anterior and barrel cortex, significant sex differences ($P=0.008$) and hemispheric asymmetries ($P=0.001$) in control mice were seen in layers II/III and were attenuated by the lesion, resulting in a nearly significant ($P=0.09$) sex \times hemisphere \times lesion interaction. Significant sex-dependent hemispheric asymmetries were observed in layers IV ($P=0.006$) and layer VI ($P=0.008$).

TABLE 1

Morphometric data from mice with unilateral (right hemisphere) 5,7-DHT injections at birth¹

Animal type	layer II/III L mean ± SEM	layer II/III R mean ± SEM	layer IV L mean ± SEM	layer IV R mean ± SEM	layer V L mean ± SEM
<i>anterior cortex</i>					
male control	192.3 ± 5.3 ^{*^}	184.0 ± 4.3 ^{*^}	201.0 ± 6.9 [^]	213.7 ± 8.6	254.9 ± 4.6 [*]
male lesion	204.0 ± 7.8	206.1 ± 7.1	214.3 ± 9.4 [^]	219.7 ± 7.6	261.3 ± 4.9
female control	237.3 ± 7.5 ^{*^}	214.8 ± 8.8 ^{*^}	249.9 ± 10.5 ^{*^}	216.9 ± 6.6 [*]	276.3 ± 8.8
female lesion	219.6 ± 7.8	213.7 ± 11.3	250.4 ± 7.1 ^{*^}	230.3 ± 8.6 [*]	261.7 ± 3.9 [*]
<i>barrel cortex</i>					
male control	176.6 ± 4.5 ^{*^}	<u>166.6 ± 3.2^{*^#}</u>	213.7 ± 4.4	211.9 ± 4.8	235.8 ± 4.0
male lesion	181.1 ± 3.7 ^{*^}	<u>174.1 ± 3.2^{*^#}</u>	221.0 ± 4.0	215.8 ± 4.6	235.7 ± 5.4
female control	190.9 ± 2.3 ^{*^}	<u>180.6 ± 5.0^{*^#}</u>	234.1 ± 5.1	<u>212.2 ± 4.7[#]</u>	265.8 ± 5.6
female lesion	193.4 ± 3.1 [^]	<u>188.4 ± 3.9^{*^#}</u>	231.7 ± 6.4	<u>234.1 ± 6.5[#]</u>	246.9 ± 5.6
<i>posterior cortex</i>					
male control	153.4 ± 5.3 ^{*^}	133.6 ± 4.6 ^{*^}	168.3 ± 6.2 ^{*^}	142.5 ± 5.9 [*]	196.4 ± 6.0
male lesion	146.5 ± 2.6 [*]	127.9 ± 2.5 ^{*^}	161.1 ± 5.8 ^{*^}	138.5 ± 6.5 ^{*^}	194.3 ± 4.8
female control	<u>117.0 ± 6.3^{*^#}</u>	150.0 ± 3.7 ^{*^}	<u>135.3 ± 4.0^{*^#}</u>	<u>153.0 ± 8.5^{*^#}</u>	<u>218.8 ± 4.5[#]</u>
female lesion	<u>158.4 ± 5.3^{*^#}</u>	160.4 ± 6.3 [^]	<u>175.9 ± 9.6^{*^#}</u>	<u>185.9 ± 6.6^{*^#}</u>	<u>186.3 ± 7.2[#]</u>
	layer V R mean ± SEM	layer VI L mean ± SEM	layer VI R mean ± SEM	total ctx L mean ± SEM	total ctx R mean ± SEM
<i>anterior cortex</i>					
male control	272.8 ± 5.4 [*]	359.0 ± 9.0 ^{*^}	341.4 ± 6.5 [*]	1076.2 ± 16.6 [^]	1091.6 ± 19.6 [^]
male lesion	279.9 ± 4.9	355.8 ± 8.7 [*]	331.3 ± 7.9 ^{*^}	1110.8 ± 29.0 [^]	1124.9 ± 23.7
female control	268.8 ± 10.0	392.2 ± 8.7 ^{*^}	333.1 ± 11.0 [*]	1250.5 ± 18.5 ^{*^}	1137.7 ± 14.2 ^{*^}
female lesion	283.6 ± 7.7 [*]	364.8 ± 12.2	361.1 ± 8.1 [^]	1179.4 ± 17.1 [^]	1172.8 ± 28.6
<i>barrel cortex</i>					
male control	234.8 ± 5.2	327.4 ± 4.7 [^]	275.6 ± 6.7 ^{*^}	1047.1 ± 14.2 ^{*^}	<u>990.4 ± 17.5^{*^#}</u>
male lesion	231.8 ± 5.9	305.7 ± 6.0 [*]	295.3 ± 6.0 [*]	1053.2 ± 13.7 ^{*^}	<u>1015.4 ± 12.9^{*^#}</u>
female control	258.7 ± 4.8	330.9 ± 7.6 [^]	296.0 ± 6.4 ^{*^}	1112.0 ± 16.8 ^{*^}	<u>1025.3 ± 13.6^{*^#}</u>
female lesion	254.6 ± 6.9	325.4 ± 9.7 [*]	311.7 ± 5.4 [*]	1098.6 ± 18.4 ^{*^}	<u>1097.3 ± 10.6^{*^#}</u>
<i>posterior cortex</i>					
male control	184.0 ± 6.4	215.8 ± 9.3 ^{*^}	151.3 ± 5.5 [*]	779.5 ± 20.8 ^{*^}	<u>674.4 ± 14.2^{*^#}</u>
male lesion	190.9 ± 5.1	200.5 ± 13.1 ^{*^}	147.9 ± 3.5 ^{*^}	763.8 ± 18.7 [*]	<u>723.4 ± 14.0^{*^#}</u>
female control	<u>228.0 ± 1.9[#]</u>	176.2 ± 5.9 ^{*^}	156.0 ± 5.7 [*]	<u>678.5 ± 27.7^{*^#}</u>	<u>751.3 ± 7.6^{*^#}</u>
female lesion	<u>207.3 ± 5.6[#]</u>	174.5 ± 7.3 [^]	171.8 ± 4.5 [^]	<u>756.5 ± 28.2[#]</u>	<u>800.9 ± 19.4^{*^#}</u>

indicates significant lesion effect (P<0.05)

* indicates significant sex differences (P<0.05)

^ indicates significant hemisphere differences (P<0.05)

¹All measurements are given in micrometers. Note the ubiquitous sex and hemisphere differences and their frequent interactions with the lesion effects. Also note significant effects in the left hemisphere (contralateral to lesion) in posterior cortex in layers IV, V, and in total cortex.

TABLE 2

Summary diagrams of the lesion effects by cortical layers in bilaterally (A) and unilaterally (B) lesioned mice

A: Bilateral 5,7-DHT lesion effects

	anterior cortex			barrel cortex			posterior cortex		
	male	female	both	male	female	both	male	female	both
total cortex	↑	0.07*	↑	↑	↑	↑	ns	ns	↑
II+III	↑	ns	↑	ns	↑	↑	ns	ns	↑
IV	0.08*	ns	↑	ns	ns	↑	ns	ns	↑
V	↑	ns	↑	ns	ns	↑	ns	↑	↑
VI	↑	↑	↑	ns	ns	0.06*	ns	ns	ns

B: Unilateral 5,7-DHT lesion effects¹

	anterior cortex			barrel cortex			posterior cortex		
	male	female	both	male	female	both	male	female	both
total cortex	ns	ns	ns	↑	↑	↑	↑	↑	↑
II+III	ns	ns	ns	↑	↑	↑	ns	↑	↑
IV	ns	ns	ns	ns	↑	↑	ns	↑	↑
V	ns	ns	ns	ns	ns	0.09*	ns	↓	ns
VI	ns	ns	ns	ns	ns	ns	ns	ns	ns

*P value (approaching significance)

¹For unilaterally lesioned mice, only effects in the right hemisphere (ipsilateral to lesion) are indicated. Upward arrows indicate significant ($P \leq 0.05$) increases in width and downward pointing arrows indicate significant decreases in width. Note that despite overall significant effects for both male and female mice, the main effect is often due to changes in one sex only.

DISCUSSION

In this study, for the first time, the effect of focal intra-parenchymal, neonatal 5,7-DHT injections on the morphogenesis of several different regions of the dorsal somato-sensory cortex have been quantified. We demonstrated that in BALB/c ByJ mice, relatively transient, neonatal mono-aminergic depletions of the cerebral cortex substantially alter cortical morphogenesis. The morphological changes, characterized largely by a significant increase in cortical layer width, displayed regional, layer, and sex-specific differences. The effects described here are fundamentally different from those resulting from neonatal electrolytic lesions that are aimed at the cholinergic basal forebrain, which largely decrease cortical layer widths (Hohmann & Berger-Sweeney, 1998b). The data show that monoaminergic depletions alter cortical morphogenesis and suggest that monoaminergic and cholinergic denervation of the neocortex results in different, and perhaps opposite, effects.

Both unilateral and bilateral injections of 5,7 DHT into the medial forebrain bundle region significantly decreased monoamines in cortical regions ipsilateral to the lesions, which resulted in increases in cortical widths. Bilateral lesions showed a more pervasive impact on cortical widths that is likely due to a more substantial monoamine, especially serotonin, depletion. This result may be particularly relevant for the anterior cortex, which displays the most striking differences between unilaterally and bilaterally lesioned mice. The anterior cortex ordinarily has the highest serotonergic innervation density and thus, only the bilateral lesions may have resulted in sufficient depletions to elicit a significant morphological response. Although we examined here monoamine levels only in unilaterally lesioned mice, our previous report showed that bilaterally lesioned mice sustain a more substantial monoamine depletion into adulthood (Berger-Sweeney et al., 1998). Furthermore,

hemispheric asymmetries might have obscured significant alterations in unilaterally lesioned mice. The results obtained in unilaterally lesioned mice revealed that monoamine depletion, in many instances, abolished the sex and hemispheric differences in cortical regions, suggesting moreover, that monoamines may be involved in establishing sex differences in cortical structure.

We hypothesize that the morphogenetic consequences of the 5,7-DHT lesion are predominantly due to serotonergic depletion of the developing neocortex. Although the lesions transiently depleted both cortical serotonin and NE, the reduction in serotonin levels were substantially larger. Whereas in the current study we examined monoamine levels in unilaterally lesioned animals, the data from our previous behavioral report attest to a comparable pattern of serotonin vs. NE depletion in bilaterally lesioned mice (Berger-Sweeney et al., 1998). Furthermore, most reports of NE depletions in the developing neocortex describe neuropil reductions, more compatible with a reduction in cortical width (Brenner et al., 1985; Felten et al., 1982; Kolb & Sutherland, 1992; Loeb et al., 1987; Onteniente et al., 1980; Osterheld-Haas & Hornung, 1996). Recent preliminary studies from our own laboratory support this view by showing that, at least in males, joint noradrenergic and dopamin-ergic depletions of the developing cortex, with 6-hydroxydopamine, result mostly in decreased cortical layer widths (Alatishe & Hohmann, 2000).

Reconciling our data with previous results of serotonin depletions in the developing cortex is difficult because the lesion parameters and the paradigms studied differ substantially. Most previous studies have employed systemic depletions of serotonin via peripheral or ventricular injections of toxins, or synthesis inhibitors (Blue et al., 1991; Bennett-Clarke et al., 1994; Mazur et al., 1997; Rhoades et al., 1998; Turlejski et al., 1997; Vitalis et al., 1998). Because serotonin apparently can

modulate transmitter release and activity in a variety of cortical afferents (Crespi et al., 1997; Dinopoulos et al., 1997; Gobert et al., 1998; Mansour-Robaey et al., 1998; Matsumoto et al., 1999), such systemic depletions may precipitate alterations in other neurotransmitter systems and could lead to secondary effects on the cortex. In contrast, in the current study 5,7-DHT was injected directly into the medial forebrain bundle fibers and thus, presumably, depleted cortically and hippocampally projecting monoaminergic fibers more selectively. Moreover, the present study represents the first time that neonatal serotonin depletions have been quantitatively compared throughout the dorsal sensory-motor cortex of adult rodents of both sexes.

Several previous studies have measured the effects of neonatal serotonin depletions on the developing barrel field cortex (Blue et al., 1991; Bennett-Clarke et al., 1994; Rhoades et al., 1998; Turlejski et al., 1997; Vitalis et al., 1998). Although the decreased barrel field size reported in those studies may, at first glance, appear in contradiction with the present results, it should be kept in mind that (1) previous data were obtained approximately 1 to 2 week postnatal, before cortical neurons and their afferent connections have fully matured, and (2) the size of the barrel field may not reflect the actual width of cortex and its layers but rather the relative functional space established by afferent thalamocortical fibers in the PMBS area. For example, neonatally nBM lesioned mice exhibit decreases in cortical layers IV and V width in the barrel field areas as adults, but do not show decreases in barrel field size at PND 8, when, on the other hand, significantly reduced barrel plasticity can be observed in response to whisker removal (Hohmann & Berger-Sweeney, 1998b; Nishimura et al., 1999).

The results of several previous studies suggest that 5-HT and acetylcholine have opposing effects on cortical development and later on cognitive

processes (Bear & Singer, 1986; Carli et al., 1997; Gu & Singer, 1993; Gu & Singer, 1995; Matsukawa, 1997; Roerig & Katz, 1997; Roerig et al., 1997). These modulatory afferents may provide precisely timed go/no-go signals to developing cortical neuritic processes. The data presented here following serotonergic depletions, when compared with data from our previous studies following cholinergic depletions (Hohmann & Berger-Sweeney, 1998b), provide support for this hypothesis; cholinergic depletions generally decrease cortical widths, whereas serotonin depletions generally increase cortical widths. Nevertheless, the interactions between these modulators is likely to be more complex than a simple opposition effect.

Interactions between serotonin and sex dimorphisms in the cortex are not without precedence (Alonso et al., 1991; Flemming et al., 1986; Steward & Kolb, 1988; Steward, 1991). Notably, in most previous studies, perinatal stress leads to alterations in serotonergic transmission and reduces the sexual dimorphism in cortical widths that is seen normally and hemispheric laterality (Flemming et al., 1986; Steward & Kolb, 1988; Steward, 1991). Behavioral effects of similar manipulations are also sex-specific (Alonso et al., 1991). The sex-, hemisphere-, and region-dependent responses to 5,7-DHT depletions, noted in the current study, suggest that different cortical neurons, in different layers and different brain regions, respond idiosyncratically to serotonin. Serotonergic responses in the cerebral cortex are mediated by a variety of different receptor molecules, of which 5-HT_{2A}, 5-HT_{1A} and B, and 5-HT₃ are the most prevalent and most extensively studied. In the adult rodent, these receptors have a widespread distribution postsynaptically on cortical pyramidal neurons and GABAergic interneurons, as well as presynaptically on afferents, including thalamocortical, cholinergic, dopaminergic, and noradrenergic fibers (Bloom & Morales, 1998; Crespi et al., 1997; Hamada et al., 1998; Heider et al., 1997; Kia et al., 1996; Marek &

Aghajanian, 1999; Matsumoto et al., 1999; Pompeiano et al., 1992). While comprehensive ontogenetic studies of these different receptors remain to be performed, it stands to reason from current data that receptors may be differentially distributed on neuronal elements in the cortex, and that such differential distribution may be the reason for the region- and layer-specific effects observed after 5,7-DHT injections (Mansour-Robaey et al., 1998). In the dentate granule cells of the hippocampus, for instance, developmental 5-HT depletions lead to decreased spine densities, and this effect is due to a lack of 5-HT_{1A} receptor activation (Haring, 1991; Faber & Haring, 1999). Likewise, inactivity of other receptor populations in the cortex may result in an increase of growth in aspects of the neuropil.

Postsynaptically, the activation of serotonergic receptors reduces cortical excitability, predominantly via the inhibition of pyramidal cells and via the activation of GABAergic interneurons (Roerig & Katz, 1997; Zhou & Hablitz, 1999; Bloom & Morales, 1998). Furthermore, cortical 5-HT_{2A} and 5-HT_{1A} receptors are modulated by sex steroids (Cyr et al., 1998; Fink et al., 1996; Flugge et al., 1998; Osterlund et al., 2000; Sumner & Finke, 1998; Zhang et al., 1999). The 5-HT_{1A} receptor appears to be down-regulated by estrogenic activity (Flugge et al., 1998; Osterlund et al., 2000; Zhang et al., 1999), whereas the 5-HT_{2A} receptor appears to be up-regulated by estrogenic activity (Cyr et al., 1998; Fink et al., 1996; Sumner & Finke, 1998). Additionally, sex differences in monoamine levels, including serotonin, have been reported in the developing and adult rodent brain (Carlsson et al., 1985; Valencia-Sanchez et al., 1997); Wilson and Agrawal, 1979). The serotonergic system can display vigorous sprouting in response to adrenergic denervation (Blue and Molliver, 1987). Although our measured values of 5-HT versus NE depletions do not favor this explanation, we cannot exclude the possibility that post-lesion serotonergic

fibers sprouting may account for the layer and region dependent variations in the cortical response to the 5,7-DHT lesion. Taken together, the data suggest that serotonin, along with other afferent neuromodulators, is involved in sculpting the neuronal substrate of the developing neocortex into adult morphology, with its subtle architectonic variations. These afferents also may modulate hormonal influences on cortical organization throughout development and maturity.

The substrate of cortical width increases remains to be elucidated. A variety of studies on neural plasticity suggest that afferent neuromodulators, including serotonin, affect dendritic growth and synaptic densities, as well as neuronal soma size in a manner that is highly specific to neuronal populations (Azmitia & Azmitia-Whitaker, 1991; Foehring and Lorenzon, 1999; Haring, 1991; Hohmann & Berger-Sweeney, 1998a; Kolb & Wishaw, 1998; Lauder, 1993; Mattson, 1988). Increases in any or all of these features could result in altered cortical widths. In addition, developmental enrichment effects, which appear to be under monoaminergic control (Benloucif et al., 1995; Pappas et al., 1987; Pappas et al., 1992) also affect glial growth and proliferation (Sirevaag & Greenough, 1991). Glial cells carry 5-HT_{1A} receptors and may mediate some of the neurotrophic effects of serotonin (Azmitia, 1999). In light of recent insights into life-long neurogenesis, an increase in the cortical neuronal population in the affected layers also should be considered (Gage et al., 1998; Kemperman & Gage, 1999). Serotonin depletions in the adult, however, appear to decrease rather than to increase neuronal proliferation (Brezun & Dazuta, 1999).

We assume that the alterations described here in neocortical development following focal neonatal 5,7-DHT lesions are in part responsible for the behavioral alterations that we previously observed after comparable bilateral lesions (Berger-Sweeney et al., 1998). In the previous study, we demonstrated

that neonatally lesioned mice had an improved performance in a delayed non-match-to-sample odor discrimination task, a deficit in 24-h retention in a passive avoidance paradigm, and no difference in open field activity or a simple odor discrimination task. Even though these mice still displayed mono-aminergic (predominantly serotonergic) deficits at the time of behavioral testing, behavioral effects were more pronounced than would be expected from the levels of neurochemical depletion; in particular, until now, passive avoidance deficits were not attributed to mild serotonin depletions. In our previous studies concerning neonatal cholinergic depletions, aimed at the basal forebrain (nBM), we described significant correlations between decreased cortical layer IV and V widths and spatial water maze acquisition deficits, in the absence of passive avoidance deficits or odor discrimination deficits (Arters et al., 1998). In contrast, after neonatal 5,7-DHT lesions, we see passive avoidance retention deficits and increased response times in a complex odor discrimination task. The same lesions result in expanded cortical width affecting all cortical neuronal layers. Male and female responses to neonatal 5,7-DHT lesions were highly diverse in a region- and layer-dependent fashion; this result is in contrast with results from neonatal electrolytic nBM lesions in which sex dimorphisms were limited to cortical layers II/III. It is somewhat surprising that we did not observe sex differences in passive avoidance or olfactory discrimination after a comparable lesion. Perhaps the behavioral tasks were not sensitive enough to detect sex differences. Alternatively, the cortical width increases following neonatal 5,7-DHT lesions are not as relevant to learning and memory performance as one might be led to hypothesize, based on the correlation data from our neonatal nBM lesion paradigm.

The constellation of altered cognitive responses following neonatal 5,7-DHT lesions have suggested

alterations of attentional mechanisms in such animals and/or impaired response inhibition, which refers to interrelated processes that permit a delay in the decision to respond (Barkley, 1997). The behavioral patterns are evocative of alterations in human disorders, including ADHD and autism (Barkley, 1997; Leekam et al., 2000; Sigman, 1998). Furthermore, autism is associated with increased cortical widths and serotonergic imbalances (Caper & Courchesne, 2000; Chugani et al., 1999; Cook & Leventhal, 1996; Deb & Thompson, 1998; Piven et al., 1996). In particular, Chugani et al. recently demonstrated decreased serotonin synthesis in the thalamic and cortical areas of a sample of autistic boys, suggesting altered serotonin modulation of thalamocortical connectivity (Chugani et al., 1997). The results of that study also suggested the presence of sex differences. Thus, the neonatal 5,7-DHT lesion paradigm we describe here has the potential to serve as a useful animal model for the study of autism and ADHD.

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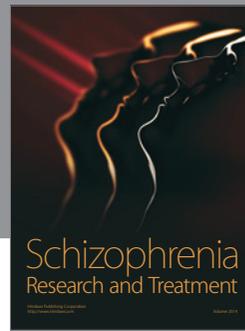
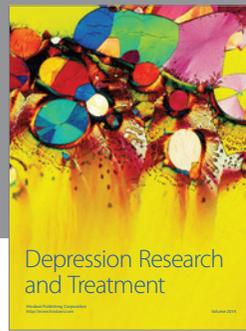
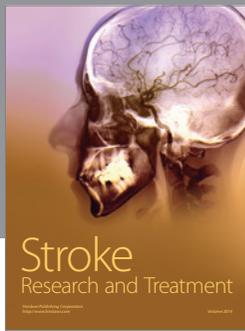
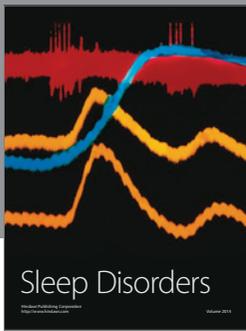
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