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Modeling Early Cortical Serotonergic Deficits in Autism

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Abstract

Autism is a developmental brain disorder characterized by deficits in social interaction, language and behavior. Brain imaging studies demonstrate increased cerebral cortical volumes and micro- and macroscopic neuroanatomic changes in children with this disorder. Alterations in forebrain serotonergic function may underlie the neuroanatomic and behavioral features of autism. Serotonin is involved in neuronal growth and plasticity and these actions are likely mediated via serotonergic and glutamatergic receptors. Few animal models of autism have been described that replicate both etiology and pathophysiology. We report here on a selective serotonin (5-HT) depletion model of this disorder in neonatal mice that mimics neurochemical and structural changes in cortex and, in addition, displays a behavioral phenotype consistent with autism. Newborn male and female mice were depleted of forebrain 5-HT with injections of the serotonergic neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT), into the bilateral medial forebrain bundle (mfb). Behavioral testing of these animals as adults revealed alterations in social, sensory and stereotypic behaviors. Lesioned mice showed significantly increased cortical width. Serotonin immunocytochemistry showed a dramatic long-lasting depletion of 5-HT containing fibers in cerebral cortex until postnatal day (PND) 60. Autoradiographic binding to high affinity 5-HT transporters was significantly but transiently reduced in cerebral cortex of 5,7-DHT-depleted mice. AMPA glutamate receptor binding was decreased at PND 15. We hypothesize that increased cerebral cortical volume and sensorimotor, cognitive and social deficits observed in both 5-HT-depleted animals and in individuals with autism, may be the result of deficiencies in timely axonal pruning to key cerebral cortical areas.

Keywords

Autism; serotonin; glutamate; plasticity; social behavior; receptor autoradiography

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Introduction

The core symptoms of autism, (i.e. - stereotypical behaviors, impaired verbal and nonverbal communication and blunted social interaction) are accompanied by structural and functional changes in cortex, cerebellum and amygdala [3,4,7,8,19–22,37,38,49,55,67,101,125]. Structural and functional changes apparent in early childhood suggest that autism is a disorder of brain development [4,19,20,37–39,111]. Several lines of evidence suggest that serotonin (5-HT) may play a pivotal role in the pathophysiology of autism. We hypothesize that perturbations in the forebrain serotonergic system, in particular, are responsible for many of the neuroanatomic and behavioral features of this disorder. An animal model that investigates the mechanisms that bring about these changes will help us better understand the neurobiology of autism. Past work from our laboratories focused on cortical development and plasticity within the context of afferent neuromodulatory systems. This paper reports on a neonatal mouse selective serotonin depletion model that we designed to mimic both the neurochemical and structural changes described in cerebral cortex of individuals with autism.

Alterations in serotonergic innervation and tone are evident in autism. Using positron emission tomography (PET) imaging of a tryptophan analogue, Chugani and colleagues [27] demonstrated that young children with autism do not display the developmental peak in whole brain 5-HT synthesis capacity seen in typically developing children. Boys with autism have specific decreases in 5-HT synthesis in the dento-thalamocortical pathway with simultaneous increases in the contralateral dentate cerebellar nucleus [28]. The abnormal patterns of cortical 5-HT synthesis capacity observed in children with autism are related to hemispheric language specialization since children with decreased 5-HT synthesis in the left cortex tended to have the most severe language impairment, while those children with right-sided or non-asymmetric patterns of cortical 5-HT synthesis had less severe language deficits [24]. Multiple studies have reported increased serum 5-HT levels, later identified at platelet-derived 5-HT, in patients with autism [2,32,35]. Pharmacotherapy with serotonergic agents that reduce CNS 5-HT generally worsen autistic symptoms [84] while those that increase available 5-HT via the high affinity 5-HT transporter (SERT) improve some autistic features [34]. Although somewhat variable, a number of recent reports indicate an association between variations in the gene that encodes SERT and a susceptibility to autism [33,43,112]. Whitaker-Azmitia and colleagues [66,123] propose a perinatal hyperserotonemic animal model for the pathophysiology of autism. They suggest that the excessively high 5-HT from the blood of the growing fetus crosses the forming blood brain barrier, gains access to the developing brain, and ultimately causes a loss of 5-HT terminals in cerebral cortex via a negative feedback system mediated by 5-HT receptors. Treated rat pups from their studies display “autistic-like” behaviors with impaired auditory, social and sensory functions, as well as brain metabolic abnormalities [66]. This model would reconcile a predisposition for increased peripheral 5-HT with central nervous system 5-HT deficits in early postnatal development in autism.

Serotonin is involved in neuronal growth and plasticity [71,124] and it likely exerts a modulatory role on cortical synaptic plasticity via regulation of glutamate receptors during both normal development and in disease states with serotonergic dysfunction. In addition, serotonin has a trophic influence on the morphogenesis of somatosensory cortex (SI) and the development of patterned glutamatergic thalamocortical connections [26,41,74,99]. Alterations in cortical 5-HT levels affect the patterning of thalamocortical maps, such as in rodent barrel field [11,16,23]. Moreover, Djavadian et al. [45] report that 5-HT depletion in neonatal cats reduces pruning of callosal axons to visual cortex, resulting in a doubling of labeled projection neurons while maintaining their overall topography. Disruption of serotonergic transmission via receptor blockade and 5-HT depletion reduces plasticity in kitten visual cortex [53,119]. Additionally, long-term 5-HT depletion with para-chlorophenylalanine (pCPA) in adult rats altered the density of alpha-amino-3-hydroxy-5-methyl-4-isoxazole

propionic acid (AMPA) glutamate receptor subunits (i.e. increased GluR2/3 and GluR2 and decreased GluR1 subunits) in membrane homogenates of cerebral cortex [106]. Interestingly, a decreased density of AMPA glutamatergic receptors also has been described in postmortem human autistic brains [96]. These findings, coupled with that of decreased 5-HT synthesis in autism, supports a role for both serotonergic and glutamatergic systems in the pathology of this disorder.

A number of structural abnormalities have been described in the brains of individuals with autism. Affected children are born with small to normal sized brains, but they then experience rapid and excessive brain growth within the first years of life resulting in increased cortical volume at 2 to 4 years of age [38,111]. This abnormal growth slows so that by late adolescence, brains are the same size as unaffected adults [3]. The findings of early postnatal brain overgrowth in autism followed by an abrupt cessation of growth in childhood, were recently confirmed in a meta-analysis of all eligible reports examining head circumference, magnetic resonance imaging (MRI), or postmortem brain weights from autism cases [97]. Initial MRI evaluation indicated that the increased brain volume in children with autism is the result of increased cerebral cortical gray matter and cerebral and cerebellar white matter [20,38]. However, more recent studies have demonstrated consistent increases in white matter volumes in children with autism [6,56–58], which may be more pertinent to the key deficits in autism involving processing of complex social, sensory and language information.

Cortical cytoarchitecture is also disturbed in autism. Postmortem microscopic evaluation of brain tissue from adults with autism demonstrates abnormalities in cerebral cortical development that include increased cortical thickness and neuronal density, neuronal disorganization related to abnormal migration, poorly differentiated gray-white matter boundaries and an increased number of white matter neurons [4]. In addition, frontal and temporal cortical minicolumns are smaller and more numerous and less compact than those of control subjects [21,22]. Alterations in the size and cellular distribution within cortical minicolumns in autism may reflect disturbances in the processing of thalamic inputs to cortex. A recent MRI study of adolescents and adults with autism demonstrated thalamic volumes that were significantly smaller than their increased brain volume would predict, which may suggest altered thalamocortical integration [115]. Spectroscopic analysis of regional brain chemical concentrations in 3 to 4 year old children with autism revealed decreased levels of N-acetylaspartate (NAA), a marker of neuronal integrity, in frontal and parietal white matter, cingulate and thalamus compared to typically developing children [50]. Thus, structural pathologies in autism cortex are consistent with observations from animal studies of altered cortical serotonergic innervation and suggest that altered 5-HT tone and altered brain development in autism may be causally related.

In our previously published study, mice with selective neonatal cortical 5-HT depletions, via injections of the serotonergic neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT) into the medial forebrain bundle (mfb), had significantly increased cortical width [60]. This is similar to the increased cortical volume described in children with autism. Changes in cortical width in mice are sex, region and layer specific. The mouse forebrain 5,7-DHT lesion prevents the characteristic developmental peak in cortical 5-HT, much like that described in young children with autism [27,28]. Thus, this selective neonatal cortical 5-HT depletion model is ideally suited to test our hypotheses that decreased neonatal 5-HT innervation can alter cortical morphogenesis and glutamatergic development and plasticity in ways that are consistent with the neuropathology in autism. Moreover, this mouse model allows us to assess the range of behavioral changes that may be directly attributed to such cortical changes. It may also expand investigations into additional brain structures that are affected directly or indirectly by serotonergic deficits in development.

While it is difficult to design animal behavioral tests, particularly in non-primates, that accurately reflect the symptoms seen in humans with autism, strong inferences can be made from species specific study of categories of behavior, such as gross motor function, social interaction, sensory processing, and repetitive/stereotypic and phobic behaviors. We review here data from previously published and ongoing studies that demonstrate that mice depleted of 5-HT after birth display a number of sex-dependent behavioral deficits that suggest a phenotype substantially compatible with the core symptoms of autism [13]. In addition, we demonstrate the selectivity and temporal impact of the neonatal depletion and give preliminary evidence that AMPA receptor development is altered after the neonatal cortical 5-HT depletion. Finally we will discuss our hypothetical model of how such neurochemical and structural alterations in development might bring about the pathologic features of autism.

2. Materials and Methods

2.1. Animals and Surgery

Unless otherwise specified, BALB/CbyJ mice for all studies were bred and housed at the Morgan State University Animal Facility according to the Institutional Animal Care and Use Committee. Animals were maintained on a 12 hour light-dark cycle and were allowed *ad libitum* access to food and water. Animals were weaned at four weeks of age and then housed two to four per cage in same sex groupings. Behavioral testing began at three months postnatal age. Immunocytochemical and autoradiographic analysis were performed at variable ages as stated below. Pups of both sexes from each litter were randomized to become 5,7-DHT-lesioned mice or sham-operated, saline-injected vehicle controls henceforth referred to in the figures as “vehicle”. In addition, un-operated, normal controls, closely matched for age (time of gestation/birth) were used in all recent studies for which we show data in this paper, henceforth referred to in the figures as “age normal”. Sample sizes for experiments not previously published are stated in the Results section.

For the lesion surgery, mouse pups were removed from their mothers within 12 hours of birth and were anesthetized with hypothermia by placing them on ice until all movement ceased (approximately 1–2 minutes). Bilateral injections of 5,7-DHT (Sigma-Aldrich; 0.6 microliter of a 5 micogram/microliter solution) or sterile saline (vehicle-injected shams) were made into the mfb as described previously (see Figure 1) [13,60]. Lesion coordinates were as follows: entry of the cannula was at 1mm anterior to the fronto-nasal suture and 1.5 mm lateral to the midline. The cannula was advance at a vertical angle of 58.5 degrees and a lateral angle of 15 degrees and injections were made upon retraction at depth of 4.0 mm, 3.5 mm and 3.0 mm. Age normal controls did not receive any injection or hypothermia anesthesia. Animals were re-warmed on a heating pad and returned to their home cage until sacrifice on postnatal days (PND) 7, 15, 30 or 60 for 5-HT immunocytochemistry or for autoradiographic binding to serotonin reuptake transporters (SERT), AMPA or N-methyl-D-aspartate (NMDA) glutamate receptors or until 8 to 12 weeks for behavioral testing.

2.2 Behavioral Testing (8 to 12 weeks postnatal age)

2.2.1. General Neurological Assessment—Mice from all treatment groups underwent a battery of neurological tests to assess coordination, balance and strength. Mice were scored with the examiner blind to treatment group. All tests were performed in triplicate and average scores for each animal were used for analysis. All mice used in the open field object recognition (OFOR) experiments, social transmission of food preference (STFP) task and cued/contextual fear conditioning (CCFC) experiments were subjected to the neurological test battery immediately before testing in these tasks. Mice were scored on the following tasks: righting reflex (to assess impairments in proprioception or equilibrium), placing reflex (to test for gross sensorimotor impairments) grip strength (gross assessment of muscle strength) and beam

walking. For the latter task we measured both the amount of time, up to a maximum of 125 seconds, taken by each mouse to cross the beam and enter the home cage as well as the ability of the mouse to cross smoothly (on a scale of 1–4) without losing grip with 2 or more paws. Mice that did not cross to their home cage by 125 seconds or fell off the beam more than twice were removed from the task and given the maximum score of 4. The beam-walking test assessed both fine motor coordination and anxiety (reluctance to cross the beam). Statistical significance was determined using factorial ANOVA with Fisher post-hoc tests (StatView).

2.2.2. Social Transmission of Food Preference (STFP) Task—This task was used to test for social learning ability as well as to assess specific indicators of social interactions between the “demonstrator” and “observer” (5,7-DHT, vehicle and age normal) mice. Mice were trained to eat powdered chow and conditioned to handling prior to behavioral testing. Female “demonstrator” mice were allowed to consume flavored food for 30 minutes and then placed into a wire-mesh enclosure. Following an 18 hour period of food deprivation, “observer” mice (male and female 5,7-DHT-lesioned, vehicle-injected shams and un-operated age normal control mice) were placed into the cage with the demonstrators and allowed to interact for 20 minutes. Interactions were videotaped and analyzed using Observer VideoPro® (Noldus) software. The following behaviors of the observer mice were quantified: location in enclosure (quadrants 1, 2, 3 and 4), nuzzling (nose to nose contact between observer and demonstrator), genital sniffing, self grooming, digging, chewing bedding, chewing wire enclosure, rearing and walking, tail rattling, and attack charges. To test for acquisition of food preference, observer mice were presented with a two-way food choice immediately after the “flavor demonstration” and, once more, 24 hours later. Consumption of the cued (demonstrated) food vs. the non-cued (new flavor) food was measured. Data in Figure 7 were generated from eight 5,7-DHT-lesioned male and nine lesioned female mice and seven age normal control male and female mice each. Statistical significance was determined using factorial ANOVA with Fisher post-hoc tests (StatView).

2.2.3. Cued/Contextual Fear Conditioning (CCFC)—This task tests both affective and cognitive aspects of behavior but uses an aversive motivator (mild foot-shock) rather than a neutral motivator as in the open field object recognition task; or a positive, (food/social contact) motivator as in the social transmission of food preference task. The behavioral measure is passive (freezing) rather than active (exploration). The CCFC task tests the animal’s ability to integrate complex sensory stimuli (the different interiors of the 2 test chambers) for the performance of a motor response (freezing). This task was performed, as adapted from Crawley [40], using the TSE Fear Conditioning System for small animals. With this equipment, the timing of stimuli and the measurement of behavioral responses are automated and computerized.

On Day 1 of testing, mice were placed into a chamber with a metal floor that could be electrified. The animals were given 2 minutes to explore the environment and freezing bouts were measured every 10 seconds. After that, the auditory conditioning stimulus (white noise, 80 dB) was sounded for 30 seconds; overlapping in the last second with a mild foot-shock of 0.035 mA. The number of seconds spent freezing (total immobility except for respiration) was measured as an indicator of the unconditioned fear response of mice prior to onset of the foot-shock. Following a 2 minute pause, the tone was sounded, once again, and freezing was measured as indicator of the conditioning effect. Twenty-four hours later (Day 2), the mouse was placed into a second test chamber which differed from the first in sensory context (smell, shape, floor texture) and freezing behavior was scored every 10 seconds for 5 minutes. At the end of the 5 minute period, the tone was sounded again for 30 seconds and the freezing response measured. In order to test the contextual discrimination of fear conditioning, the same mouse was returned on Day 3 into the original conditioning chamber for 5 minutes and freezing response was measured once every 10 seconds. The number of freezing bouts on Day 2

compared to Day 3, was regarded as a measure of contextually conditioned fear. In normal mice, freezing should be decreased in the novel context (unconditioned environment) compared to the conditioning environment. We compared all freezing responses to sound on Days 1, 2 and 3 among 5,7-DHT, vehicle and age normal groups as well as freezing responses with and without sound in the two different environments. Cued conditioning was calculated as the number of freezing bouts in the conditioning environment compared to the number of bouts in the novel environment with and without the auditory cue. Figures 8 and 9 represent the evaluation of 17 5,7-DHT-lesioned male and female mice and 28 male and female age normal control mice. Vehicle injected mice did not significantly differ from age normal controls in post-hoc analysis but displayed slightly lower freezing, under all conditions, in males. Statistical significance was initially determined using a factorial ANOVA (StatView) with Fisher post-hoc tests. Significant differences between male 5,7-DHT and male age normal controls were further verified using t-tests.

2.2.4. Open Field Object Recognition (OFOR)—This task was adapted from a task developed by Ricceri et al. [100] in accordance with parameters introduced by Poucet [95]. The OFOR task was performed in a round enclosure of approximately 3 feet in diameter, surrounded by walls of about ten inches high that lean outward at a slight angle of about 15 degrees (commercial plastic baby pool). The entire enclosure was painted black to increase visibility of the white mice; quadrants and annuli demarcating periphery versus center were marked on the floor with white lines. Mice were videotaped during the performance of all aspects of the task and all measurements were conducted on a Dell Pentium computer using Observer VideoPro® (Noldus) software. The objects referred to below consisted of Lego constructions of various shapes and colors. The apparatus was wiped with an alcohol/water solution between subjects to eliminate all possible odor cues. Objects constructed of Lego blocks were placed in the testing arena as described below. Mice were placed into the enclosure for 6 minutes with 3 minute intervals between sessions. The experimenter recorded the time spent with each object.

In the first session, mice were placed into the enclosure without objects to assess open field behaviors including locomotor activity (number of quadrant crossings), rearing and time spent exploring the perimeter versus the center of the field (indicators of anxiety level). Anxiety scores were calculated by dividing time spent in the periphery by time spent in the center. During sessions 2 through 4, 5 objects of different shapes and colors were placed at set locations in the enclosure and the amount of time a mouse spent exploring (whisking, sniffing, climbing on or exploring with forepaws) the objects was scored. In sessions 5 through 7, objects were displaced or objects were replaced with a novel object and the same exploratory behaviors were scored. The reported study included 9 male and 7 female 5,7-DHT-lesioned mice and 6 male and 12 female vehicle-injected control littermates. Statistical significance was determined using factorial ANOVA (StatView) with Fisher post-hoc tests.

2.2.5. Additional behavioral tests—For a detailed description of the passive avoidance task, the simple odor discrimination task and the delayed none matched to sample odor discrimination task, please consult our paper by Berger-Sweeney et al. [13]. Please note that for these initial studies all mice were raised and tested at Wellesley College in the laboratory of Dr. Berger-Sweeney. However, as for all other experiments, the lesion and sham surgeries were performed in the laboratory of C. Hohmann at Morgan State University, according to the methods described above.

2.3. Serotonin Immunocytochemistry

Animals from each treatment group were deeply anesthetized with either hypothermia (PND 7 and 15) or with chloral hydrate (0.1 ml intraperitoneal; 300 mg/ml solution; PND 30 and 60)

and perfused transcardially with 0.9% saline in 0.1 M sodium phosphate buffer (pH 7.4) followed by ice cold 4% paraformaldehyde. Volume and pump speeds varied depending on the age of each animal. Animals were decapitated and their brains carefully removed from the skull. After 4 to 6 hours post-fixation (longer times for younger animals) in the same paraformaldehyde solution, brains were cryoprotected in a 30% sucrose solution and stored at -20°C prior to sectioning. Brains were cut on a freezing microtome into 50 μm parasagittal sections at PND 7 and 40 μm sections for PND 15 and older mice; sections were collected sequentially into ice-cold PBS buffer in a twelve-well tissue culture plate. Every fourth section from each animal was rinsed twice in phosphate buffered saline (PBS) and processed for the immunocytochemical detection of 5-HT according to previously described methods [46]. The density of serotonin immunoreactive axons was measured in parasagittal sections at PND 7, 15, 30 and 60 as described by Mamounas et al. [82] in frontal, parietal and occipital cortex, hippocampus, thalamus and cerebellum (Figure 1). Three to seven animals per treatment group (age normal control, and 5,7-DHT-lesioned) at PND 7, 15 and 30 were analyzed statistically. Density values for each region and age were compared between normal and 5,7-DHT-lesioned mice by t-tests. These analyses and the graphs generated were composed using GraphPad Prism. Values are shown as mean $\pm$ standard errors (s.e.m.).

2.4. Cortical Morphometry

Paraformaldehyde-fixed tissues for cortical morphometry were obtained from PND 90 animals from each treatment group. As described in Hohmann et al. [60,61], quantitative morphological assessments were performed on 50 μm coronal sections that were stained with Methylene Blue-Azure II (Sigma-Aldrich). Briefly, using the MCID, AIS imaging system (Imaging Research, Inc., St. Catherine's, Ontario) images of the tissue were digitized and cortical width was measured at two separate levels through the anterior and posterior somatosensory cortex. In each section, bilateral cortical and laminar thickness measurements were taken at four separate medial to lateral positions within the somatosensory cortex. Means of these individual measurements for each section were used for statistical analysis using an analysis of variance (ANOVA) (StatView).

2.5. Autoradiography

Animals from each treatment group were deeply anesthetized, killed by rapid decapitation and their brains quickly removed and frozen on dry ice. Brains were stored at -80°C prior to sectioning. Tissues were cut into 20 μm thick sections on a cryostat set at -20°C , thaw mounted onto Superfrost Plus slides (Fisher Scientific) and stored at -80°C in plastic-covered microscope slide boxes. For each animal, adjacent sections through the forebrain and midbrain were used to examine autoradiographic binding to SERT or to AMPA and NMDA glutamate receptors in cerebral cortex. Six animals at each age, PND 15, 30 and 60, and one section per animal were used for analysis. Tissues were stained with cresyl violet after x-ray film exposure to delineate cellular elements and anatomic boundaries. Autoradiographic binding to the SERT was performed using [^3H]-citalopram (Amersham Biosciences), as described previously [10, 16,18]. AMPA-sensitive binding sites were labeled with [^3H]-AMPA (Amersham Biosciences) as described by Brennen et al. [17] and NMDA-sensitive binding sites were labeled with [^3H]-CGP (Amersham Biosciences) as described by Jaarsma et al. [63].

Autoradiographic films were analyzed by densitometric analysis using a video based image analysis system (Imaging Research, Inc., St. Catherine's, Ontario). A set of ^3H standards was exposed with the tissue sections for quantification of binding density. A relative optical density value was measured for each standard and these were then fit to an interpolation function. In these studies, the best fit was provided by a second degree polygonal function. Using this function, optical densities of the autoradiographic sections were compared to densities produced by the [^3H] standards and transformed to nCi/mg tissue. The Nissl-stained sections

were helpful in delineating the cerebral cortical boundaries. Six animals per treatment group (age normal control, vehicle-injected sham, and 5,7-DHT-lesion) at PND 7, 15 and 30 were used for analysis. Statistical analyses performed included one and two way (age versus group) ANOVA with Tukey's multiple comparisons test. These analyses and the graphs generated were performed using GraphPad Prism. Values are shown as mean $\pm$ standard errors (s.e.m.).

3. Results

3.1. Serotonin Immunocytochemistry

Our neonatal, focal 5,7-DHT lesions resulted in a substantial (85%) selective depletion of the cortical and hippocampal 5-HT levels in early postnatal development followed by recovery towards adulthood [60]. This neonatal rodent model was designed to mimic the reduction in forebrain 5-HT levels described by Chugani and colleagues in children with autism [27]. When looking directly at the serotonergic fibers by means of 5-HT immunohistochemistry (see Figure 2), neonatal 5,7-DHT lesions produced a near complete loss of 5-HT immunoreactive neurons in cerebral cortex and hippocampus at PND 7. At all ages, 5-HT positive fibers were clearly visible in their normal distribution and density in brain regions unaffected by the mfb lesion, such as deeper forebrain subcortical regions, midbrain, and brainstem. As shown in Figure 3, bilateral lesions to the mfb with 5,7-DHT on the day of birth result in significant reductions in 5-HT immunoreactivity in most regions of cerebral cortex and hippocampus until PND 60 as compared with both vehicle and age normal groups (* $p < 0.05$, ** $p < 0.01$). A few of the PND 30 and PND 60 mice showed regenerative sprouting of 5-HT containing fibers in frontal cortex. Such a substantial, lasting decrease in 5-HT fibers seemed inconsistent with our previous HPLC finding showing only a 30% (statistically not significant) reduction in cortical 5-HT levels by adulthood. However, this discrepancy could be explained by a post-lesion increase in serotonin transporter sites beginning with adolescence. Our autoradiographic study of SERT sites showed that the density of SERT sites in 5,7-DHT-treated mice was reduced in both cortex and hippocampus until PND 15 [61]. However, at PND 30 the 5,7-DHT-lesioned mice showed an increased density of uptake sites as compared with age normal controls but not with vehicle-injected animals. The elevation in cortical 5-HT uptake site expression in the 5,7-DHT-lesioned mice over time thus may compensate for the 5-HT loss in these animals, normalizing 5-HT neurotransmission in adulthood.

3.2. Glutamate Receptor Autoradiography

Glutamate receptor density in cerebral cortex was altered following neonatal selective forebrain 5-HT depletion. Results of autoradiographic binding to glutamate receptors showed that with age, the density of AMPA and NMDA receptors in cerebral cortex increased significantly ($p < 0.0001$ for age; see Figures 4 and 5). AMPA receptor binding density in 5-HT-depleted animals was significantly decreased in cerebral cortex at PND 15 as compared with vehicle-injected and age normal controls ($p < 0.05$; Figure 6). A two way ANOVA for age versus group that evaluated all groups and ages showed near significance for a group effect ($p = 0.057$ for group; Figure 6), although densities did not vary significantly at individual ages. NMDA receptor binding density was unchanged in 5,7-DHT-depleted animals at PND 7, 15 or 30. For NMDA receptors, a two way ANOVA suggested a trend for group differences, when the entire cohort was analyzed ($p = 0.10$ for group). The disruption of 5-HT innervation to cortex, mediated through 5-HT and glutamatergic receptors, may underlie the increases in cerebral cortical width and behavioral alterations in mice that resemble findings seen in individuals with autism. We will further discuss this hypothesis in the Discussion.

3.3. Behavioral Characterization

As summarized in Table 1, selective neonatal forebrain 5-HT depletion caused sex-dependent impairments in social, repetitive and sensorimotor behaviors that were akin to those seen in

autism. At the same time, however, functions such as spatial learning and memory, gross motor performance and general locomotor activity were unimpaired as they are in many subjects with autism, particularly higher functioning individuals. Anxiety responses in our mice appear to be contingent on changes in the environment and are not generalized, another analogy to the core symptoms of autism.

3.3.1. Impaired Impulse Inhibition—We have previously published behavioral studies that suggest impaired impulse inhibition in the neonatally lesioned mice [13]. When tested on a passive avoidance task, 5,7-DHT-lesioned males and females acquired this aversively motivated task normally, but when tested 24 hours later, lesioned males, in particular, displayed a significant performance deficit (50% decrease in latency compared to age matched normal and vehicle control males). The passive avoidance task trained the mice to avoid the preferred, darkened side of an enclosure by exposing them to a mild foot-shock upon entering the dark compartment [40]. Latencies to enter the dark compartment were measured as indicators of task acquisition/retention. It usually took just two acquisition trials (separated by one hour) with the aversive stimulus (foot-shock) to significantly increase the latency of the test subjects to re-enter the dark. This increased latency persisted in normal mice when they were retested 24 hours later. It should be noted however, that even age normal control mice re-entered the dark compartment eventually (after several minutes).

Increased latency in the 24 hour phase of the passive avoidance task may be interpreted as a retention deficit. However, this would stand in clear contradiction to normal odor discrimination performance and even slightly superior performance (compared to vehicle and age matched normal controls) on a delayed non-match to sample odor discrimination task that was displayed by the same 5,7-DHT-lesioned cohort of mice [13]. In the simple odor discrimination task, the mice learned to associate a buried food reward (chocolate chip in a cup of sand) with a particular scent. After this, in the delayed non-match to sample task (DNMS), mice learned to associate the food reward with the cup that did not carry the scent to which they were initially exposed. The presentation of the training scent and choice samples were separated by a 30 second delay. Arguably, the DNMS task required more sophisticated learning and memory skills than the passive avoidance task. Therefore, the 24 retention deficit in the lesioned males in the passive avoidance task may not be due to a memory problem at all, but rather the consequence of impaired ability of the lesioned mice to inhibit the impulse to enter the dark compartment. Poor impulse inhibition also could account for the significantly shorter latencies to retrieve the food reward in the DNMS task in lesioned compared to control mice, although it does not account readily for their fewer errors. The odor discrimination studies also taught us two additional facts about our 5,7-DHT-lesioned mice: a) they appeared to have normal odor discrimination ability and b) food reward was a very good motivator for them. We employed these properties subsequently in the implementation of a social food preference task as a paradigm for the detection of possible social deficits (see below). Our initial behavioral studies showed normal nocturnal activity levels in the neonatally the 5,7-DHT-injected mice, suggesting that acute adult 5-HT deficits are not likely involved in the other behavioral deficits [81]. Normal locomotor activity and gross motor function were corroborated in different tests for basic locomotor function.

3.3.2. General Neurological Testing—A basic neurological examination was performed on all subsequent cohorts of mice (see methods) in order to see if complex task performance might have been influenced or biased by basic sensory or motor impairments. Adult mice with neonatal 5,7-DHT injections showed no gross sensory or motor deficits compared to age normal control and vehicle-injected sham mice. However, male 5-HT-depleted mice displayed impaired fine motor performance in that some could not cross a small round beam without losing their footing. However, this trend was not statistically significant for the group. Fine motor deficits are also a hallmark of autism [69,73,85,91,92,104,113,120].

3.3.3. Open Field Object Recognition (OFOR) Testing—The open field paradigm was the next logical choice for a task to assess exploratory activity together with a new cognitive dimension (spatial learning and memory), alongside a means to assess general anxiety levels and responses to novelty. The OFOR data were recently submitted for publication elsewhere and thus will only be summarized here. Introduction of a novel objects into the maze environment provoked a significant anxiety response in male lesioned mice. However, the same mice showed only a non-significant trend for increased anxiety in terms of open field exploration, prior to object placement in the OFOR task. Moreover, change in object positions, after the mice had established a “routine” of exploring the same 5 objects in the same locations for three six minute sessions, resulted in a significant 30% decrease ($p=0.024$) of all exploratory activity in lesioned male mice. Changing object position is ordinarily used to assess spatial learning and memory and normal mice increase exploration, particularly of the displaced objects [95,100,103]. The results of the OFOR analysis showed that lesioned mice acquired and retained spatial information relatively normally, but had an abnormal response to this challenge that suggested aversion to spatial change.

3.3.4 Social Transmission of Food Preference (STFP) Task—In order to address the most fundamental deficit of autism, it was clearly necessary to test our mice for their ability to perform socially with other mice. Rather than purely assess the animals’ desire to associate with a conspecific or not, we tested performance in a social transmission of food preference (STFP) task. This task allowed us to assess the subject’s ability to learn information from a conspecific and simultaneously assess features of the animal’s interaction with the conspecific. As described in the methods, mice (male or female, lesioned or vehicle and age matched normal control observers) acquired a cued food scent from a normal, adult, female demonstrator mouse. The observer mouse was placed in a spacious enclosure with the demonstrator for 30 minutes. The demonstrator, who had consumed a “cued” flavor was confined to a small wire cage in one end of the enclosure but the observer could roam about, interact or not with the demonstrator, or even climb on top of the demonstrator’s enclosure. We found to our surprise that the lesioned male and female mice did not avoid the demonstrator at all, and spent as much time as vehicle-injected controls in all the quadrants of the enclosure. They also had similar locomotor activity and climbing and rearing behaviors. However, the quality of interaction with the demonstrator was altered in the lesioned mice. In particular, male lesioned mice engaged in significantly less ano-genital sniffing of the female demonstrators ($p<0.05$, ANOVA main effect compared to vehicle and age normal controls) and on several occasions aggressively charged the cage with the female demonstrator without any previous warning. We did not assess the estrous state of our female demonstrators but the behavior of our age normal control males (tested on the same days) did not suggest that the females were in estrous at the time of the encounter and none of the vehicle-injected or age normal control males displayed aggression towards the female demonstrators. Thus, male lesioned observer mice clearly displayed a qualitatively abnormal social approach towards the female demonstrator mice. Lesioned mice also showed significant increases in bouts of self-grooming ($p<0.05$, ANOVA main effect compared to both vehicle and age normal controls), which is akin to the stereotypies and other repetitive behaviors in autism. Thus, the neonatally 5,7-DHT-lesioned males clearly displayed inappropriate social behavior in their interactions with the demonstrators. Lesioned mice also displayed an altered frequency in digging in the bedding compared to vehicle controls ($p=0.05$, Fisher posthoc).

After 30 minutes of social interaction time, mice were tested for acquisition of the demonstrated “cued” food flavor, by presenting them with two cups of differently flavored food (one cued, one new flavor) and measuring food consumption. Not surprisingly, the altered quality of social interactions did interfere with the ability to acquire the cued food preference (see Figure 7). Interestingly, preferential consumption of the cued, demonstrated food was disrupted significantly in both male and female lesioned mice. Since normal female mice (in our hands)

consumed more cued food than males, the disruption in female consumption appeared even more dramatic than in the lesioned male mice. Subsequent preliminary data suggested that flavor pairing (cued/non-cued) influenced relative food consumption in both vehicle and age matched controls and lesioned mice alike. However, the performance of 5,7-DHT-lesioned mice differed from controls, no matter what flavors were presented. We are currently analyzing additional data in this paradigm.

3.3.5. Cued and Contextual Fear Conditioning (CCFC)—Deficits in the processing of sensory information are a key symptom of autism. Responses to auditory signals, in particular, are often altered in individuals with autism. Thus, we implemented the CCFC task in a non-traditional manner, to assess the ability of lesioned mice to appropriately respond to an auditory conditioned stimulus and to use multi-sensory (visual, olfactory and tactile) information to identify a cued environment. Although the CCFC task is most commonly used to quantify fear responses and is associated strongly with hippocampal and amygdala circuitry [40,75], cortical function is also involved in appropriate performance of this paradigm [86,114].

Our data showed that mice depleted of 5-HT at birth did not respond appropriately to either sound or contextual (multi-sensory) cues. As described in detail in the Materials and Methods section, the freezing (fear) response of a mouse to tone was measured first without negative conditioning stimulus (mild foot shock) and subsequently in conjunction with the foot shock. Male lesioned mice failed to respond to the sound prior to its pairing with foot-shock. Thereafter, the 5,7-DHT-treated mice displayed an exaggerated response to sound, suggesting altered sensory processing and sensory-emotional integration (see Figure 8). In contrast, female lesioned mice performed similarly to age matched normal controls. Lesioned mice also responded differently to the environmental cues, depending on their sex. Normal mice associated the conditioning tone with the environment in which it occurred. Consequently, they displayed fewer freezing response in a novel environment on Day 2 than when they were re-exposed to the conditioning environment on Day 3. In contrast, the serotonin-depleted males actually increased their freezing response in the novel environment on Day 2 relative to that on Day 3 when they were re-exposed to the conditioning environment (see Figure 9).

4. Discussion

We report here on a rodent model of autism that replicates a number of neurochemical, behavioral and structural features of the human disorder. Despite the inherent difficulties in designing animal experiments that appropriately represent human behaviors, and in comparing animal and human behaviors, our findings show a compelling resemblance to several core symptoms of the autism phenotype. Mice that receive neonatal injections of the serotonergic neurotoxin, 5,7-DHT, into the major afferent pathway to the forebrain, the mfb, show significant reductions in the density of 5-HT containing fibers in cerebral cortex and hippocampus. These reductions persist in an attenuated form into adulthood. Our data also show that mice depleted of 5-HT at birth have sex-dependent deficits in processing social, sensory and environmental information as adults when compared with sham-lesioned littermates (vehicle group) and age-matched normal mice (age normal group). Perhaps most striking, 5-HT-depleted mice demonstrate significant increases in the width of cerebral cortex that are region, layer and sex-specific. These increases are also age dependent, much like the expansion of cortical volume in imaging studies of children and adults with autism. These data support our hypothesis that some of the behavioral features of autism are rooted in early developmental alterations in cortical morphogenesis that are influenced by its serotonergic innervation.

As described previously, children with autism have significantly increased cortical volumes at 2 to 4 years of age, as the result of an early, exuberant brain overgrowth [20,38]. Cortical

volume normalizes with increasing age into adulthood [97] and, depending on the report, may be smaller in later life [102]. Similarly, at PND 90, the lesioned mice showed robust increases in the width of all cortical layers, with different layers affected to a different extent in males and females [60]. However, at approximately one year of age, only layer IV was still significantly increased in width and overall cortical size was no longer different from controls [61]. Moreover, our recent preliminary data suggests that substantial cortical volume increases exist as early as PND 7 (unpublished observations). These results suggest that forebrain neonatal 5-HT depletion causes permanent morphologic changes in the structure of the cerebral cortex, and these developmental alterations are evident within one week of serotonergic manipulation, at PND 7. This age in mouse is similar, in terms of cortical maturation, to the youngest children with autism who have increased cortical volumes [36–38,111].

The disruption in 5-HT innervation to cerebral cortex that occurs in autism, likely has profound effects on cortical morphogenesis and subsequent behavior repertoires. We suggest here that altered forebrain 5-HT innervation, acting via 5-HT and glutamate receptor sites, disrupts the appropriate pruning of cortical axons and plasticity of cortical maps. The role of 5-HT as a morphogen in cerebral cortical development is well described [71,124], as are the interactions of 5-HT and glutamate on neuronal plasticity [72,79,80]. For example, application of 5-HT to cultured neonatal thalamic neurons caused neurite outgrowth and elaboration [79,80]. Serotonin also increased the proportion of glutamate containing neurons in embryonic cortical slices without affecting the proportion of other neuronal or glial cell types [72]. *In vitro* electrophysiologic studies demonstrated that exogenous application of 5-HT produces enhanced excitatory NMDA-evoked responses in rat [98] and cat [89] neocortical slices. Rodent studies *in vivo* showed that 5-HT was present during critical periods of postnatal rat [41,78,90] and mouse [59] cortical development and shows a more prevalent peak in male BALB/CbyJ mice [31]. Interestingly, a similar early postnatal peak is also evident in typically developing human cortex [24].

Serotonin also is involved in the development of patterned thalamocortical connections in rodent SI. Afferent inputs from the whiskers or vibrissae of multiple rodent species form a highly organized and topographic map from the periphery to brainstem, thalamus and finally to layer IV of SI [14,48,54,68,105,108,116,121,127]. In the early postnatal period, serotonin immunoreactivity, 5-HT_{1B} receptors and SERT each assume a transient pattern localized to layer IV thalamocortical axons [9,10,12,18,26,41,74,76,83,99]. Multiple studies have revealed that manipulation of early 5-HT levels results in significant alterations in the layer IV representations of the rat's whiskers [11,15,16,23,117]. These findings all support a modulatory role for 5-HT on the development and patterning of the thalamocortical innervation, which uses glutamate as its transmitter.

It is now well understood that activation of NMDA and AMPA glutamate receptors is essential for activity-dependent stabilization of synapses and refinement of cortical maps, and that the development of these maps depends on a careful balance of excitatory and inhibitory input to the brain from sensory information and endogenous activity (see [30,44,64,93] for review). Excitatory synapses that are activated are retained, as are their axons and target neurons, while those that are less active are lost. In typically developing humans for instance, the absolute number of cortical synapses doubles from birth to 2 years of age but then steadily declines during childhood to early adulthood [62], as excess synapses are removed via activity-dependent plasticity. Loss of these synapses and their projection axons, also referred to as pruning, is an essential part of typical brain development. Without such pruning, sensory maps remain ill defined and abnormal sensory perception and sensorimotor processing ensues [29].

In support of a role of 5-HT in the regulation of synaptic pruning, studies by Djavadian et al. [45] have shown that 5-HT depletion in neonatal cats reduces pruning of callosal axons to

visual cortex. In these studies, neonatal cats depleted of 5-HT with 5,7-DHT had twice as many neurons project across the corpus callosum in visual cortex at 3 months of life, compared to control cats. These authors concluded that 5-HT enhanced developmental plasticity by increasing axonal pruning. Thus, depletion of cortical 5-HT may reduce plasticity, by stabilization of projection fibers that normally would have been eliminated. In our model, the neonatal 5,7-DHT-lesion reduced available cortical 5-HT resulting in increased cortical thickness. We hypothesize that the width increase in the 5-HT deficient mice is due to diminished pruning. Moreover, the decreased density of AMPA receptors in the cerebral cortex of 5-HT-depleted mice at PND 15 suggests an overall decrease in cortical activity. Decreased activity likely interferes with the stabilization of synapses and thus the refinement of cortical maps in a particularly critical time in development [14,105,116,127].

We propose that reductions in available cortical 5-HT during infancy may underlie some of the histopathological features of autism. Casanova et al. [21,22] showed that in postmortem brain tissues from individuals with autism, the frontal and temporal cortical minicolumns were smaller, more numerous and less compact than those of non-autistic subjects. Alterations in the size and cellular distribution within cortical minicolumns in autism may reflect disturbances in the processing of thalamic inputs to cortex. These findings are consistent with the increased cortical volumes described in children with autism. MRI studies have shown that the increased brain volumes in children with autism are to a large degree the result of increased white matter volumes [6,56–58]. Using MRI-base morphometric analysis Herbert and colleagues [57] report disproportionately larger cerebral white matter volumes in boys with autism. They have gone on to localize the increased white matter volume to later or longer-myelinating regions of the radiate white matter compartment to all cerebral lobes (frontal, parietal, temporal and occipital) [58]. Akshoomoff et al. [1] showed that in very young children with autism, variations in cerebral and cerebellar volumes, particularly white matter volumes, are correlated with diagnostic and functional outcomes. These findings are also consistent with decreased synaptic and axonal pruning. Although, the number of neurons may also be reduced during the pruning process in cortex, the main effect is a reduction of axonal afferents. These are originally connected to multiple targets and with maturation prune and fine tune their connections to innervate a reduced target area [29,30,44,62,65,93,110]. We presently do not know whether increases in gray or white matter compartments account for the increased cortical thickness observed in our 5-HT-depleted mice but we are currently conducting stereological assessments to address this question.

Behaviorally, autism is defined by an inability to properly relate to other humans and also characterized by altered sensory perception and sensorimotor processing. Some have hypothesized that altered perception and sensory-motor processing may, in fact, be at the root of altered social behaviors [5,25,92,122]. This idea may be further supported by studies showing that early multi-modal sensorimotor stimulation can ameliorate core deficits in children with autism [69]. Absence or insufficient pruning at the appropriate times in development could lead to abnormal sensory maps and sensorimotor processing, and there are multiple studies showing alterations in sensory maps in autism. Levitt and colleagues found altered cortical sulcal maps in children with autism [77]. Such changes in the architecture of important cortical processing areas could underlie the functional deficits in the development of cortical sound processing [51], visual-motor maps [88] and face recognition [42,94] that are observed in autism.

The behavioral deficits, brought about by impaired 5-HT tone during critical developmental periods in our mouse model, are akin to those described in humans with autism without mental retardation [47,52,70,109]. The lesioned mice had no obvious gross motor or locomotor deficits but males had a subtle indication of a fine motor control problem as is reported in autism

[69,73,85,91,104,113,120]. The lesioned mice had good associative and spatial learning and memory ability but nevertheless, displayed cognitive deficits on multiple tasks.

Male 5,7-DHT-treated mice, in particular, showed altered responsiveness to changes in their environment (see Table 1). In the CCFC task, male lesioned mice failed completely to respond to the sound cue the first time it was issued and showed a stronger fear response in the non-conditioned, novel environment compared to the conditioning environment. Thus, either the lesioned males were unable to differentiate the novel from the conditioning environment or, more likely, they were able to distinguish the environments quite well but their “desire for sameness” actually made them fear the novel environment more than the noxious but familiar one. Inability to distinguish the novel from the conditioning environment more likely would have resulted in comparable amounts of freezing in both environments. Moreover, the second interpretation of the data is consistent with our observations in the OFOR task where displacement of objects, as well as introduction of a new object, elicited a neophobic response characterized by diminished exploration. In addition, the lack of an appropriate conditioned response to the cued environment is reminiscent of our earlier observations of a 24 hour retention deficit in the passive avoidance task. Jointly, these data point to an impairment in the process of translating sensory experience into an emotionally and functionally appropriate motor response, following neonatal serotonergic depletion in male mice. It is unclear what the underlying brain circuitry for these deficits may be, particularly as our model selectively depletes dorsal cortex (including frontal cortex) and hippocampus but not the amygdala of serotonergic innervation. However, this model will allow us to characterize the development of the specific neuropathologies that may precipitate this behavior, which is relevant for understanding autism.

Both male and female 5,7-DHT-treated mice were impaired in the learning and retention of a socially acquired task, the STFP. During the acquisition phase of the task, lesioned mice displayed more repetitive behaviors and lesioned males, in particular, displayed inappropriate social approach. It stands to reason that the qualitatively different social interactions influenced the acquisition of the subsequent food preference since lesioned mice did not show a statistically significant increased consumption of cued vs. non-cued food. Interestingly, overall food consumption during the testing phase was also reduced although the lesioned mice always display normal body weight compared to vehicle and age normal mice and also consumed comparable amounts of non-flavored powdered chow during the training phase.

Finally, our previously published studies demonstrate that male 5-HT-depleted mice exhibit indications of impaired impulse inhibition as compared with intact animals. This was despite having normal or even improved learning on discrimination tasks and normal learning and memory abilities in a two-way discrimination task.

The persistence of sex differences in neonatal serotonin lesion effects is likely another important component of understanding the neuropathology of autism. With the exception of the socially transmitted food preference task, where food consumption was impaired in both sexes, male neonatally 5,7-DHT-lesioned male mice were affected uniquely or more markedly than female lesioned mice. This may be highly relevant in regards to autism where, by most accounts, four times as many males as females are diagnosed [87,107,118,126]. Our own developmental observations in male and female mice [31] together with the data from Chugani's group [27,28] suggest that magnitude of an early postnatal peak or elevation in cortical serotonergic innervation is sex specific. Thus, disruption of this early transient elevation may be affecting male cortical development more profoundly than female cortex. Alternatively, there may be differences in timing of the serotonergic developmental signal in male and female brains as has already been indicated for the development of the cholinergic modulatory influence to cortex [31,60].

Another recent rodent model lends support to our hypothesis of the role played by early serotonergic innervation in the etiology of autism. Although Whitaker-Azmitia and colleagues [66,123] propose a hyperserotonemic model of autism, their hypothesis is ultimately consistent with our own. In their studies, perinatal rat pups were exposed to a serotonergic agonist from mid-gestation to PND 20. However, as a result of autoregulation via 5HT_{1A} receptors, the excessive 5-HT levels actually produced a loss of 5-HT terminals and subsequent alterations in cortical development. Rats in these studies displayed a number of social and sensory deficits and metabolic brain abnormalities that were similar to those described in children with autism. Specifically, their rats showed an increase in calcitonin gene-related peptide (CGRP) in the amygdala and a decrease in oxytocin immunoreactivity in hypothalamus at PND 45, which these authors conclude may be due to a loss of the 5-HT innervation from raphe to these brain regions.

5. Conclusions

In conclusion, this mouse model of autism possesses both face and construct validity. In terms of face validity, our results feature multiple, independent deficits in behavioral categories relevant to the autistic phenotype. Our model also exhibits construct validity in that it is based on one neuropathology prevalent in autism, an early decrease in the serotonergic innervation to cortex. This depletion results in a second feature of autism, increased cortical size. Further studies in this model will investigate the neurobiology underlying altered behaviors and determine in which ways impaired pruning and plasticity contribute to disrupted cortical connectivity and function. We plan to further probe the construct validity of this model by investigating the effect of 5-HT lesions on development and plasticity of cortical modularity using the whisker-to-barrel system. We anticipate that the outcome of these studies will prove beneficial in designing strategies for treatment.

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Figure 1. PND 7 age normal control

This figure illustrates the pattern of 5-HT immunostaining in a parasagittal section. Overlaid are labels of the different brain regions that the raphe serotonergic cells project to: frontal cortex (fr ctx), parietal cortex (par ctx), occipital cortex (occ ctx), hippocampus (hippo), striatum, thalamus, midbrain and cerebellum (cblm). Although not present in this section, the approximate locations of the dorsal raphe and the medial forebrain bundle (mfb) are demarcated. Arrows illustrate the path the serotonergic axons travel from the dorsal raphe through the mfb to the cortex and hippocampus.

Figure 2. Effects of the 5,7-DHT lesion on serotonin immunocytochemistry

Bilateral lesions to the mfb with 5,7-DHT on the day of birth produced near complete loss of 5-HT immunoreactivity in cerebral cortex and hippocampus at PND 7, 15 and 30 but did not affect the distribution of these fibers in brain regions unaffected by lesion. An arrow points to the remaining 5-HT staining in layer IV of barrel field cortex in lesioned mice at PND 7. This is the site where thalamocortical axons transiently take up serotonin at this age.

5-HT Innervation Density

Figure 3. Density of 5-HT containing fibers

Densitometric analysis of 5-HT immunoreactivity showed a decreased density of 5-HT containing fibers in most regions of cerebral cortex and hippocampus in 5,7-DHT-treated mice at most of the ages studied (*p<0.05, **p<0.01). In contrast, the density of 5-HT axons was unchanged in thalamus and cerebellum after forebrain 5-HT depletion.

Figure 4. Localization of AMPA receptor sites

Representative autoradiograms of AMPA receptor binding in coronal sections from age normal and 5,7-DHT-depleted PND 7, 15 and 30 mice. Of the few AMPA sites present at PND 7, most were in the hippocampus, striatum and ventral cortex. By PND15, the density of AMPA receptors increased markedly in dorsal cortex, striatum and hippocampus. A similar pattern of AMPA receptors was present at PND 30. The look up table in the PND 15 lesioned autoradiogram indicates that cooler colors refer to lower densities of receptors and warmer colors for higher densities of receptors.

Figure 5. Localization of NMDA receptor sites

Representative autoradiograms of NMDA receptor binding in coronal sections from age normal and 5,7-DHT-depleted PND 7, 15 and 30 mice. At PND 7 very few NMDA sites present. By PND 15, NMDA receptor density increased in the cortex and hippocampus. With age, the density of these receptors increased throughout the cerebral cortex for all groups of mice.

Binding to Glutamate Receptors

Figure 6. Density of AMPA and NMDA receptor binding

The mean density $\pm$ s.e.m. of AMPA and NMDA receptors in cerebral cortex from PND 7, 15, and 30 age normal control, vehicle-injected, and 5,7-DHT-lesioned mice. AMPA receptor binding density in cerebral cortex of 5,7-DHT-lesioned mice is significantly less compared to age normal and vehicle-injected animals at PND 15 ($p < 0.05$) but was unchanged at PND 7 and 30. NMDA receptor binding density was unchanged at PND 7, 15 or 30. A two way ANOVA showed that the density of both AMPA and NMDA receptors increased with age ($p < 0.0001$). This analysis also showed a trend for a group effect for AMPA ($p = 0.057$) and NMDA ($p = 0.103$) receptors.

Consumption of Cued Food

Figure 7. Social Transmission of Food Preference Task

The amount of cued (demonstrated) flavor consumed by neonatally 5,7-DHT-lesioned males and females was significantly lower ($p=0.038$; $*p<0.05$) than in age normal control mice. Vehicle-injected mice were also tested and consumed quantities of cued food exceeding the consumption of age normal controls. Non-cued food consumption in all groups was below the measurable scale for this graph. Serotonin-depleted mice of either sex consumed approximately 50% as much non-cued as cued food. These differences were not statistically significant. However, age normal and vehicle-injected control mice consumed less non-cued food as compared with cued food ($p=0.05$).

Response to Tone: Conditioned Stimulus

Figure 8. CCFC sound

None of the male 5,7-DHT males responded with freezing bouts to the first exposure of a tone cue, while **all** age matched normal controls did. Upon pairing the sound cue with foot-shock later on Day 1, the lesioned mice did respond with freezing and 24 hours later, male lesioned mice responded with increased freezing to re-exposure to the sound cue (differences on Day 1 tone 2 were not significant, differences on Day 2 approached significance). No significant differences or trends were observed in the females.

Response to Context

Figure 9. CCFC context

Female mice, in general, showed more freezing bouts than males. All females and male age normal control mice increased the freezing response when re-exposed to the original conditioning chamber on Day 3 (compared to Day 2) and freezing responses in the cued environment significantly increased from first exposure to the conditioned stimulus on Day 1 ($p=0.0017$ for male age matched controls, $p=0.004$ and 0.007 respectively for female age matched controls and female lesioned mice). In contrast, 5,7-DHT lesioned males actually decreased their freezing response on Day 3 compared to Day 2, and had markedly lower freezing responses compared to age normal control males and showed no significant differences in freezing between first exposure on Day 1 and Day 3.

Table 1

Observation	Behavioral Test	Study
Impaired ability to withhold a strongly motivated response (impaired impulse inhibition) (mostly males)	Passive Avoidance task	Berger-Sweeney et al. '98
Altered sensory responsiveness (males only)	Cued/Contextual Fear Conditioning (auditory response)	Figures 8 and 9
Hyper-responsiveness to changes in the environment (males only)	Open Field Object Recognition task (OFOR) (object displacement and novelty) Cued/Contextual Fear Conditioning (response to context)	Hohmann et al. Brain Res. submitted;
Impaired social learning (both sexes)	Altered acquisition and retention of a socially motivated food preference task	Hohmann et al. in preparation; see Walker et al. SfN abstract 2005, IBNS '05
Increased social aggression (mostly males)	Social Transmission of Food Preference (STFP) task (attacks on demonstrator)	Hohmann et al. in preparation; see Walker et al. SfN abstract 2005
Decreased sex appropriate behavior (males only)	STFP task (decreased ano-genital exploration of female demonstrator)	Hohmann et al. in preparation see Walker et al. SfN abstract 2005
Increased repetitive behavior (both sexes)	STFP task (increased grooming and altered digging behavior)	Hohmann et al. in preparation see also: Walker et al. SfN abstract 2005
Impaired fine motor performance (males only)	Neurological Test Battery, Beam Crossing ability	Hohmann et al., Brain Res. submitted
Normal muscle strength, proprioception and basic reflexes (both sexes)	Neurological Test Battery	Hohmann et al., Brain Res. submitted
Normal locomotor activity	OFOR task, quadrant crossing (diurnal) Nocturnal activity assessments	Hohmann et al., Brain Res. submitted; Berger-Sweeney et al. '98
Normal and even improved learning on a two-way discrimination task	Simple Odor Discrimination and Delayed Non-matched-to-Sample Odor Discrimination task, respectively	Berger-Sweeney et al. '98
Normal spatial learning and memory	OFOR task, object displacement	Hohmann et al., Brain Res. submitted;