Evaluation of Motor Cortex Neuronal Morphology in Developmental Hyperserotonemia Rat Model

Colten Z. Dillinger
Missouri State University, Dillinger489@live.missouristate.edu

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EVALUATION OF MOTOR CORTEX NEURON MORPHOLOGY IN A
DEVELOPMENTAL HYPERSEROTONEMIA RAT MODEL

A Master’s Thesis
Presented to
The Graduate College of
Missouri State University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science, Cell & Molecular Biology

By
Colten Zane Dillinger

December 2017
EVALUATION OF MOTOR CORTEX NEURON MORPHOLOGY IN DEVELOPMENT HYPERSEROTONEMIC RATS

Biomedical Sciences

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Colten Dillinger

ABSTRACT

Fetal serotonin levels are involved in the development of the serotonergic system in an autoregulatory manner as well as the organization and connectivity of non-serotonergic neurons. Insufficient serotonin levels during development result in improper neuronal maturation and decreased synaptogenesis. Conversely, excess developmental serotonin levels can alter the progression of serotonergic neurons, ultimately resulting in a chronic decrease of serotonin in the developed brain via a negative feedback mechanism. There is a known correlation between autistic patients and chronically decreased brain serotonin concentrations; this is potentially implicated in the impaired development of the autistic brain. Incomplete or delayed development of motor ability is a hallmark symptom and diagnostic consideration of ASD. The association of developmental hyperserotonemia and abnormal development of the motor cortex remains largely unstudied. This study proposes a method to further analyze the morphology and connectivity of cortical neurons located in the rat motor cortex. Ballistic transfection of the carboxylphilic dye DiI and subsequent imaging using confocal microscopy allows visualization and quantification of neuronal morphology. This study employs an adapted procedure allowing staining of multiple tissue sections at once using a BioRad Helios chambered gene gun.

KEYWORDS: developmental hyperserotonemia, DiOlistic labeling, pyramidal neuron, motor cortex, dendritic arborization, dendritic spine density

This abstract is approved as to form and content

_______________________________
Lyon H. Hough, PhD
Chairperson, Advisory Committee
Missouri State University
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Approved:

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Lyon H. Hough, PhD

________________________________________
Joshua J. Smith, PhD

________________________________________
Benjamin F. Timson, PhD

________________________________________
Julie Masterson, PhD: Dean, Graduate College

In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.
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INTRODUCTION

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder resulting in decreased cognitive ability, repetitive behavior, and impaired social interaction. The spectrum includes a range of presentations with differences in the rate of development and the resulting function in the developed state. ASD patients typically exhibit delayed development in the first two years of life that may be accompanied by subsequent regression in social skills and cognitive function. The variation in areas of impairment and the severity of these impairments is accompanied by a large number of inconsistencies in genetic, environmental, and neuroanatomical factors across patients. For this reason, the etiology of the symptoms associated with ASD is not yet well-defined. Further experimental investigation of this etiology has been constructed around previously identified common findings amongst autistic patients. Currently, the most consistent finding across patients within the spectrum is an alteration in serum serotonin concentration.

Serotonin is most commonly known in the context of its role as a neurotransmitter in the central nervous system. Central nervous system serotonin is synthesized via tryptophan hydroxylase 2 (TPH2) in the raphe nuclei, located along the medial aspect of the reticular formation. Serotonin acts in the central nervous system by binding to a variety of serotonin receptors to regulate mood, appetite, and the sleep/wake cycle. Additional experimental evidence suggests that serotonin receptor activation is involved in memory formation and the facilitation of learning. In addition to neurotransmission, serotonin also serves a variety of roles in the systemic circulation. Systemic serotonin is synthesized by tryptophan hydroxylase 1 (TPH1) in the enterochromaffin cells located in
The serotonin produced in the gastrointestinal tract binds to and activates receptors located on gastrointestinal epithelial cells to stimulate peristaltic motility in response to intestinal filling. Serotonin is drained into the gastrointestinal venous system where excess serotonin is bound by circulating platelets. In both the central nervous system and the systemic circulation, serotonin is metabolized by monoamine oxidases. As with most substrates, concentration of serotonin in both the central nervous system and gastrointestinal tract is regulated by regulating the rate of synthesis and the rate of clearance. Due to the developmental nature of structures within the brain, the ability to regulate the concentration of serotonin within the brain is determined early in development. The pathways involved in the maintenance of the concentration of serotonin are similar within the central nervous system and the systemic circulation, but the two systems function separately. The separation of these two systems is established by the inability of serotonin to pass through the blood-brain barrier.

Genetic or environmental influences that affect the development of serotonergic neurons are hypothesized to result in a chronic alteration in the ability to synthesize serotonin.

Despite the various physiological changes and neuroanatomical abnormalities seen amongst patients falling within the autism spectrum, there are consistencies present. The most striking of these consistencies is a chronic 20-50% increase in whole blood serotonin and an accompanying decrease in central nervous system serotonin (5). As with the other abnormalities observed in autistic patients, it is hypothesized that these abnormal serotonin concentrations are a result of events occurring during development. Research has shown that the formation of the serotonergic system within the central nervous system is particularly sensitive to the concentration of serotonin during the
development of the serotonergic system itself. The negative feedback loop facilitated by the serotonin autoreceptor, 5HT1, is hypothesized to decrease the ability to produce serotonin in the developed brain. In this hypothetical model increased activation of 5-HT1 results in decreased proliferation of serotonin producing neurons. Once the developmental stage in which these neurons are formed has passed, these neurons are no longer produced. The end result is a chronically decreased ability to produce serotonin within the developed brain. This hypothesis is particularly relevant in the context of pharmaceutical and illicit drugs resulting in an increase in circulating serotonin. The blood-brain barrier has not completely developed during the stage of fetal development during which the serotonergic system is formed. This incomplete blood-brain barrier may allow serotonin or drugs affecting serotonin metabolism to enter the fetal brain. The resulting increase in available serotonin within the developing brain potentially disrupts the development of the serotonergic system – causing a chronically decreased concentration within the central nervous system.

The rat model used in this study employs the addition of the exogenous non-specific serotonin receptor agonist 5-methoxytryptophan (5-MT) into the maternal circulation. This has been previously shown to result in a respective increase in circulating fetal serotonin concentration (23). 5-MT doses were chosen to mimic the degree of increased serotonin seen in autistic patients.

In addition to the cognitive and communicative deficits seen in autistic patients, ASD is commonly associated with a slowed development in fine motor skills. Fine motor ability exercises, such as choreographed finger tapping, are a diagnostic tool included in the evaluation and diagnosis of ASD. Despite the correlation between poor
motor development and ASD, the effect of developmental serotonin concentrations on the formation of the motor cortex has not been quantified. This study examines the impact of developmental hyperserotonemia on the development of neurons located within the motor cortex. Changes in motor cortical neuron morphology observed in hyperserotonemic brains would strengthen the hypothesis that developmental hyperserotonemia is a mechanism that potentially explains the hallmark deficits seen in ASD.

Pyramidal neurons located in the motor cortex have a characteristic appearance involving long, highly branched dendrites. The wide area spanned by these dendrites allow for communication with a large number of neighboring neurons. Dendritic spines located along these dendrites serve as the site where projections from other neurons synapse. The spines themselves are dynamic structures with multiple possible morphologies. These morphologies are characterized by differences in diameter, shape, and length. Each spine contains a dynamic actin scaffold that provides a mechanism allowing plasticity in the spine morphology. These spines are believed to change dimensions during the maturation process. Time-lapse microscopy has shown that nascent spines are typically present in elongated states and eventually transition into mushroom-shaped spines (24). Mushroom-shaped spines, named for the relatively large synaptic surface and short stature, are hypothesized to be the mature morphology of spines due to the relative decreased likelihood of transition from this morphology to other morphologies. It is speculated that newly-formed spines elongate in search of a nearby neurite to synapse with and, once this synapse has formed, the shape transitions into a mushroom shape to increase synaptic surface area. Since these spines are the synaptic sites along each dendrite, the number of spines is directly correlated to the degree of
connectivity that each dendrite (and cumulatively each neuron) has with surrounding neurons. Quantification of individual spine morphology, total number of dendritic spines along a dendrite, and the change in spine density along the neurite gives insight to the overall connectivity of the neuron in question.

Stereological quantification of dendritic spines has been historically difficult due to inherent characteristics of the histological procedures used to stain and image neurons. Traditional staining methods such as Golgi staining require long periods of treatment and subject the tissue to harsh chemicals. This presents a threat to the integrity of neuronal membranes and makes the reliable measuring of small membranous structures difficult. Recently, the use of DiOlistic staining has become a promising method for observing neuronal projections. The procedure involves conjugation of the fluorescent carboxylipophilic dye, DiI with microscopic tungsten particles. These particles are then fired into the tissue, where the dye dissociates from the tungsten particle and diffuses along the neuronal membrane. The lipophilic nature of the compound makes the dissociation of the dye from the membrane energetically unfavorable – preventing the dye from diffusing into the intracellular fluid or the phosphate buffered saline (PBS) solution used to mount the tissue for imaging. The resulting tissue can be imaged using fluorescence microscopy to specifically visualize the membranous features of neuronal projections. Additionally, the staining procedure does not involve long incubation periods or chemicals known to damage the plasma membrane.
LITERATURE REVIEW

Serotonin is a versatile indolamine neurotransmitter critically involved with physical and psychological function during development and throughout life. Activation of serotonin receptors in the central nervous system is directly associated with mood, cognition, and behavior. The pharmaceutical manipulation of serotonin receptors and transporters has been the empirical treatment of major depressive disorder since the discovery of tricyclic antidepressants in the 1950’s. In addition to its role as a neurotransmitter, serotonin acts as a trophic factor involved in the development of the CNS. Subsequent studies have shown increasing or decreasing developmental serotonin levels results in morphological and behavioral consequences with severity depending on the degree of change (21, 5, 8). The most common neurochemical finding among autistic patients is a chronic decrease in brain serotonin levels (4), suggesting improper development of the serotonergic system is implicated in the neuropathology of Autism Spectrum Disorder.

The serotonergic neurotransmitter network is the earliest to develop in the mammalian brain, with the first serotonergic neurons appearing in humans as early as 5 weeks of development (1, 15). The urgency in the development of the serotonergic system reflects the importance in the initiation of neural development during rudimentary stages. The increase of serotonergic neuron growth continues, with projections extending to the cortex by 10 weeks and visible raphe nuclei appearing by 20 weeks of gestation (1, 10). This collection of nuclei is the primary source of serotonin production in the brain and extends projections to nearly every functional section of the brain and spinal cord.
Immunocytochemical analysis shows that serotonergic projections from early raphe nuclei neurons appear in specific cortical areas in sequential order independent of the distance of that area from the raphe nuclei (20). This suggests a preferential growth of the serotonergic projections to meet the serotonin demands of individual areas of the brain during development, indicating time of exposure as a critical factor in addition to serotonin concentration.

As the number of serotonergic neurons and the area supplied by their projections increases, serotonin concentrations increase during the development of the CNS. This increase continues in humans until serotonin levels peak at approximately 2 years after birth with a 50% decrease found by age 5 (3). The gradual increase in serotonin concentration until the age of 2 corresponds to the window of major neurological development, after which serotonin levels begin to drop to normal physiological parameters for the remainder of the lifespan. Increases in the concentration of serotonin and the number of serotonergic neurons are followed by increased expression of serotonin receptors. Interestingly, serotonin transporters begin appearing on non-serotonergic neurons around the same time serotonergic projections extend towards the cortex (20). These transporters internalize serotonin, removing it from the synapse and subsequently limiting activation of extracellular 5-HT receptors. Despite the continuous increase in serotonin concentration during development, the early presence of these transporters suggests a need for regulating receptor activity to avoid overstimulation.

5HT-1A receptors are the earliest of the serotonin receptors to emerge in the developing brain as well as the earliest to reach their peak concentration (2). During development of the serotonergic system, 5HT-1A autoreceptors located on serotonergic
neurons participate in modulation of further development by a negative feedback mechanism (15). Autocrine activation of 5HT-1A receptors by serotonin binding decreases the synaptic release of serotonin (15). Drug-induced depletion of this receptor during developing mice primarily results in fetal death (15). The proportional concentration increase of serotonin and 5-HT1A receptors combined with an observed decrease in serotonergic neuron survival in 5-HT1A knock-out mice point to the 5-HT1A receptor as a key component in the development of the serotonergic system. Depletion of brain serotonin during development via TPH2 knockout has been shown to decrease axonal elongation of serotonergic neurons in mice (27). This necessity for serotonin binding to its receptor on a neuron responsible for producing serotonin indicates that the development of the serotonergic system is reliant upon an autoregulatory process.

Serotonin levels outside the normal parameters during development may alter this process, causing abnormal numbers and abnormal morphologies of serotonergic neurons in the developed brain (22). This presents potential pathological implications both physically and psychologically.

In addition to the autoregulatory effects of serotonin in the development of the serotonergic system, it plays a role in the organization and morphology of non-serotonergic neurons. The presence of serotonin during early development has been shown to promote dendritic elaboration. Cerebellar sections taken from rats exposed to small amounts of serotonin during early prenatal development show an increased dendritic area in pyramidal neurons compared to a control. Conversely, rats exposed to small amounts of serotonin during late fetal development and rats exposed to large amounts during early development showed a decrease in dendritic area (11). The role of
serotonin in the formation and maintenance of synaptic connections in the spinal cord was demonstrated using adult and newly hatched chickens. Following injections of the serotonin receptor depleting drug parachlorophenylalanine (PCPA), the average number of synaptic connections among neurons decreased in both age groups (13).

It is evident through depletion studies that the insufficient concentration of serotonin during development disrupts growth of both serotonergic and non-serotonergic neurons. When serotonin concentration falls below the normal developmental parameters the growth of the serotonergic system is stunted along with the establishment and maintenance of synaptic connections. Conversely, elevated developmental concentrations of serotonin have pathological implications extending into adulthood (6, 11). It is hypothesized that abnormally elevated blood serotonin levels during pregnancy, particularly as a result of selective serotonin reuptake inhibitors or recreational drugs, allows increased diffusion of serotonin across the underdeveloped blood-brain-barrier (23). Hyperstimulation of serotonin receptors is believed to disrupt the autoregulatory mechanism of the serotonergic system in a negative feedback manner, reducing the number of serotonin-producing neurons during the critical period in which they develop. The ability to create more of these neurons is lost by the time of birth, resulting in a chronic hyposerotonemic state in the CNS. This mechanism is a potential origin of the low brain serotonin levels commonly presented by autistic patients (4).

Developmental hyperserotonemia has also been connected with abnormal brain morphology (11). The cerebral cortex is formed during early development primarily by radial neuronal migration. During this process, nascent neurons travel along radial glial projections from the site of proliferation in the subventricular zone to the cortical plate to
form distinct cortical layers (14). Exposure to elevated levels of serotonin during this phase of development stunts the process of neuronal migration by reducing the rate at which the neurons travel towards the cortical plate in a dose-dependent manner, with normal migration rates resumed upon washing the cells to remove serotonin (14). Exposure to a drug selectively targeting the 5-HT6 receptor elicits the same effect, suggesting that activation of this receptor by serotonin is primarily responsible for the slowing effect (14). This altered rate of migration disrupts the ability of pyramidal neurons to migrate into distinct layers, causing dysplasia of the cortical plate and improper connectivity between neurons (21). Analysis of histological sections reveals these cortical dysplasias are found at an elevated rate in the brains of human autistic subjects compared to brains of non-autistic subjects (21). Heterotopias are a more severe example of slowed radial neuronal migration resulting from a near permanent stop in outward motion creating a mass of gray matter stranded in the subventricular zone. Heterotopias are commonly associated with autism and epilepsy (21, 6), making the presence of heterotopias a potential cause for the comorbidity between the two disorders. Despite their abnormal location, the neurons in heterotopias are still capable of being stimulated and fire similarly to correctly organized pyramidal cells (3); this synchronized firing in a typically unstimulated area of the brain may be sufficient to cause a seizure. Autism-related seizures may also be directly triggered by the activation of serotonin receptors. In a study using in utero serotonin injections in a mouse model to replicate hyperserotonemic conditions, juvenile control mice and developmentally hyperserotonemic mice were injected with serotonin. Mice that were exposed to high levels of serotonin during development frequently had post-injection seizures and the mice that had not been
treated with serotonin during development had no seizures after the serotonin injection (12). It is suspected that serotonin receptors in the DHS mice had an increase in sensitivity in an attempt to compensate for low levels of brain serotonin. The seizures triggered by high doses of serotonin are likely a result of receptor hypersensitivity in DHS mice. These findings point to developmental hyperserotonemia as a potential cause of cortical abnormalities resulting in the cognitive issues associated with ASD and as a direct and indirect cause of seizure.

Autism Spectrum Disorder is characterized by a deficiency in social cognition, difficult communication, and repetitive behavior accompanied by reluctance to engage in novel behavior. The hypothesis that developmental hyperserotonemia may be an underpinning of autism is supported by mouse model studies showing consistencies in altered brain morphology as well as decreased social cognition. Mouse pups injected with non-selective serotonin receptor agonist 5-MT beginning at gestational day 12 spent significantly less time with their dams, experienced less maternal bonding, and engaged in olfactory based bonding less when compared with saline treated mice (12). Genetic modification is also used to elicit a decrease in serotonin. SERT Ala56 is a rare gene variant found in approximately 1% of the European population with increased presentation amongst autistic patients (18). The mutation in the serotonin transporter increases SERT phosphorylation, rendering it constitutively active. The constant internalization of serotonin via SERT decreases the concentration of synaptic serotonin able to activate external receptors, mimicking the low levels of serotonin commonly associated with autism. Mice transfected with this gene variant were inclined to retreat rather than assert dominance when confronted by another mouse in a straight tube and
were less likely to enter chambers containing novel mice when compared to wild-type mice (18). Recordings of the mice revealed that SERT Ala56 mice were only half as vocal among littermates as WT mice (18), showing an inhibition in the most basic form of communication.

In addition to a marked change in social behavior, the developmental hyperserotonemia mice showed an imbalance in chemicals associated with social interaction. Post mortem evaluation of brain sections taken from DHS mice showed a significant decrease in cells containing oxytocin, a hormone known to promote social interaction. The same evaluation also reported an increase of cells containing calcitonin-gene related peptide, a protein involved in the fear response due to external stimuli (12). Despite inherent difficulties in paralleling mouse and human behavior, a multifaceted analysis of mouse behavior shows a correlation between lessened serotonin receptor activation and generalized antisocial behavior. A similar reduced serotonin synthesis capacity is seen in autism (5), advancing the hypothesis that the antisocial behavior of autistic patients may be a result of insufficient brain serotonin caused by decreased numbers of serotonergic neurons.

ASD is typically accompanied by the inability to develop muscle memory and difficulty executing fine motor movement. It is hypothesized that this inhibition of fine motor skills contributes to the ineffective communication skills presented in autism patients in addition to difficulty executing locomotion. Disruption of fine motor involvement of the tongue and throat interferes with the production of distinct sounds and word formation. Additionally, the subconscious mimicking of facial expressions may be impaired due to morphological abnormalities in the cerebellar-thalamocortical tract. To
examine the effect of DHS on fine motor coordination, the cerebella of mice given prenatal injections of the non-specific serotonin receptor agonist 5-MT to replicate hyperserotonemic conditions during development were collected for evaluation. Analysis of cerebellar sections from treated mice were found to have higher numbers of dendritic branches, decreased mean branch length, and decreased mean distance from the cell body to dendritic end (8). This results in a highly branched, shorter dendritic structure with a smaller radius of interaction with other neurons. The density of dendritic spines was also significantly lower among treated mice, with the spine density decreasing as the dendrite extends radially from the neuronal body. This decrease in spine density implies a reduction in the synaptic ability of cerebellar neurons in DHS mice, particularly in the distal ends of dendrites (8). Ultimately the smaller dendritic radius and a decrease in dendritic spine density as the dendrite progresses away from the cell results in decreased long-distance connectivity in cerebellar neurons. This decreased connectivity as a result of developmental hyperserotonemia may play a role in the characteristic motor deficits of ASD.

EXPERIMENTAL PROCEDURES

General

The scope of this procedure has two main focuses: the optimization of the tissue staining procedure and the use of the optimized procedure to generate experimental data. The DiOlistic labeling technique is novel to this institution and required adaptation to the resources and equipment available. Furthermore, the parameters of the labeling process
were altered in order to reduce both the cost and time required without sacrificing the efficacy of the labeling process or the quality of the resulting images. The optimization of the staining procedure was evaluated experimentally and these parameters were used for the neuronal morphology data collection.

**Optimization of DiOlistic Staining Procedure**

The use of DiOlistic staining has primarily been conducted with specialized equipment made solely for this method of staining. These staining apparatuses use plastic tubing or modified pipette tips that are prepared by pipetting up a microcarrier solution and discarding the excess solution resulting in a coat of microcarrier particles adhered to the sides of the tube or modified pipette tip. The particles are then expelled from the tip and into the tissue at close range at an approximate pressure of 200 psi. The method of staining used in this procedure uses a standard BioRad Helios chambered helium-pressure BioListic transfection device. This presents some inherent differences in the properties of the DiI-conjugated tungsten microcarriers and their ballistic trajectory. These issues were addressed by altering the composition of the microcarrier solution, the preparation of the macrocarrier firing disks, the pressure at which the particles were delivered, and the distance at which the tissue was placed from the macrocarrier firing disk during the firing process.

The first parameter addressed was the inclusion of polyvinylpyrrolidone (PVP), a plastic polymer used to facilitate the adherence of tungsten microcarriers to the sides of the tubing or modified pipette tips, into the microcarrier solution. The macrocarriers used in the chambered gene gun are prepared by aliquoting the microcarrier solution directly
onto the plastic macrocarrier disk and allowing the solution to dry. It was further hypothesized that removing PVP from the solution would alter the binding of tungsten particles to the DiI stain itself. To evaluate this, trial microcarrier solutions were prepared using 100% ethanol as a solvent for comparison with the standard solutions prepared using dichloromethane as a solvent.

The next parameter that was altered was the distance at which the tissue was placed from the macrocarrier firing disk during the firing process. Unlike the previous methods of DiOlistic labeling that use pressures of approximately 200 psi, the BioRad Helios chambered system does not allow the tissue to be placed directly under the macrocarrier firing disk. Furthermore, the low pressures used with previous methods are unlikely to penetrate cell cultures or tissue that this system is intended to transfect. For this reason, there are no rupture disks designed to rupture at pressures this low. The protocol used in this experiment uses the standard rupture disks designed to fire at 900 psi. To accommodate this, staining trials were performed with tissue samples placed at two different distances during the firing procedure: 5.1 cm and 8.2 cm (Figure 1B). The increased distance between the macrocarrier disk and tissue samples naturally increases the radius of the tungsten microcarrier spread. For this reason, it was hypothesized that multiple sections of tissue could be stained with a single shot. The possibility of staining multiple sections with one shot without sacrificing usable tissue sections would greatly decrease the associated cost of this method and allow for larger sample sizes in the future. This possibility was evaluated by comparing the number of usable tissue sections and corresponding quantifiable neurons from sections that were stained simultaneously in groups of 1, 3, and 4 neurons (Figure 1A).
The efficacy of these protocol alterations was determined by the resulting number of appropriately stained tissue sections. A tissue was deemed usable if the stain allowed neurons that were quantifiable using confocal microscopy. Neurons quantifiable for this study are those with a visible soma and associated neurites that have clear dendritic spines with dimensions that are measurable using the image data collection program ImageJ. All tissues used in the optimization of the staining procedure were procured from untreated breeder rats obtained from the Missouri State University vivarium.

**Animals**

Three timed-pregnant Sprague-Dawley rats were purchased from Charles River (Wilmington, MA) to arrive at gestational day 12. Confirmation of pregnancy was determined at Charles River with the appearance of a vaginal plug marking gestational day 0. The dams were housed in separate clear polycarbonate cages at the Missouri State University vivarium. A repeating day-night cycle was established with the lights constantly on from 0800-2000 h. An ambient temperature of 21.5°C-24.5°C was maintained in the vivarium. All dams were housed with dry food and water available ad libitum.

**Treatment**

Two timed-pregnant rats were randomly designated to receive injection of the non-selective serotonin receptor agonist, 5-methoxytryptamine (5-MT), while the remaining rat received injections of phosphate buffered saline (PBS). 5-methoxytryptamine was chosen to simulate the hyperserotonemic conditions due to its increased stability in solution and decreased clearance rate by monoamine oxidases A and B in vivo. Additionally, the decreased rate of clearance of 5-MT allowed for a more
consistent elevation of 5-HT receptor stimulus and required less frequent injections. A
dose of 1.0 mg/kg was chosen to replicate the approximate 50% increase of serum
serotonin in autistic patients that exhibit elevated serotonin levels (Whitaker-Azmitia,
2005). Dose calculations included the decreased rate of 5-MT metabolism compared to
the metabolism of 5-HT. Control groups received volume-equivalent injections of PBS.
Injections were administered to dams subcutaneously using standard disposable insulin
needles in the nuchal region at intervals of 24 hours (at 0900 h) until birth. Subcutaneous
injections of 1.0 mg/kg 5-MT or saline were then administered to pups subcutaneously
using Hamilton 50 uL glass syringes. Pups received these injections from PND1 to the
day of sacrifice, PND20. This timeline was chosen to correlate with the timeline of brain
development with complete development occurring at PND20.

Anesthesia, Perfusion, and Surgical Retrieval of Brain Tissue

Five rats were randomly selected from both the 5-MT treatment group and the
saline-receiving control group for tissue collection. Rats were deeply anesthetized using
a 3% vaporized isoflurane and oxygen mixture. Anesthesia was determined to be
sufficient when rats no longer responded to hind paw squeeze. A nose cone emitting the
same isoflurane mixture was fixed to the nose of each rat during the procedure to
maintain sufficient anesthesia during surgery. Using surgical scissors, an incision was
made below the inferior margin of the thoracic cage and continued bilaterally along the
lateral aspect of the thoracic cage. The diaphragm was penetrated and the thoracic cage
was reflected superiorly using hemostats, exposing the thoracic cavity. A puncture
incision was made at the apex of the heart and another puncture incision was made in the
right atrium to allow complete perfusion of the circulatory system. A blunt-tipped
catheter was fitted to a 50 mL syringe and inserted into the left ventricle. Rats were transcardially perfused initially with 50 mL of 25 mM PBS to clear any blood remaining in the vessels. Sufficient PBS perfusion was determined by the clearance of the liver and pallor of the paws, nose, and tail. The catheter was left in place in the left ventricle and a syringe filled with 1.5% paraformaldehyde solution was fitted to the catheter. A second perfusion of 50 mL of 1.5% paraformaldehyde solution was then performed to fixate the tissue. Sufficient paraformaldehyde fixation was determined by stiffening of the limbs. Perfusions were performed slowly with careful attention paid to avoid pulmonary vessel rupture and leakage into the thoracic cavity. The lungs, nose, and mouth were constantly monitored to ensure that solutions were not leaking through the respiratory system. The skull was separated by making an incision through the atlanto-occipital space with a standard razor blade. A rongeur was carefully inserted into the foramen magnum and pieces of calvarium were removed in the posterior to anterior direction. When the entirety of the brain was exposed, the cranial nerves were severed from the inferior aspect of the brain to allow for removal. The brain was then placed into a 1.5% paraformaldehyde solution. Whole brains were stored in 1.5% paraformaldehyde at 4°C until sectioned within the next 12 hours.

**Tissue Sectioning**

Brains were removed from paraformaldehyde and placed into a metal cutting matrix. The motor cortex was selectively excised from the tissue by making an incision just posterior to the olfactory bulb and just anterior to the thalamus. A 4% agarose gel was prepared in a standard Erlenmeyer flask and allowed to cool until nearly solidified to prevent damaging the tissue upon contact. The tissue containing the motor cortex was
then fixed in a 4% agarose gel matrix (posterior side down) for stability while sectioning. The agarose-encased tissue was then fixed to a sectioning block (posterior side down) using cyanoacrylate adhesive. The vibratome reservoir was filled with 4°C 25 μM PBS to provide an isotonic environment for tissue immediately after sectioning. Vibratome blades were initially washed with xylene to remove the protective oil coating on the blades and then washed a second time using 100% ethanol. The cutting block and attached agarose-encased tissue were fitted into the vibratome holding apparatus. The apparatus was manually raised in increments of 150 microns and the blade was passed through the tissue at a low speed setting of 2 (10 maximum) to prevent any shearing of the tissue. Sections were removed from the PBS reservoir using a soft, fine-tipped paintbrush and placed into individual wells filled with 25 mM PBS in a 24-well plate. The plates were stored at 4°C until staining the next day.

**Conjugation of Dil and Tungsten Microcarriers and Preparation of Macrocarriers**

75 μg of tungsten beads was weighed using a mass and weigh paper and placed onto a standard glass microscope slide. A razor blade was used to “chop” the tungsten on the slide, simultaneous removing any large groups of beads and creating a uniform layer over the slide. 4.5 μg of Dil was then completely dissolved in 75 μL of dichloromethane. Using an auto-pipette, the entire Dil-dichloromethane solution was aliquoted over the tungsten beads carefully to ensure saturation of the tungsten. The mixture was allowed to dry and scraped into a 15 mL conical tube containing 9 mL of DD H2O. This solution was sonicated for 20 minutes to further reduce the size of tungsten particles in solution. Following sonication, the completed slurry was stored at 4°C until macrocarrier assemblies were prepared. BioRad Helios™ reusable steel macrocarrier holders were
removed from 100% ethanol and allowed to dry. Macrocarriers were then fitted into the carriers with minimal contact to avoid any residue collection on the carriers. The previously prepared slurry was then vortexed and 75 µL was aliquoted onto the center of each macrocarrier, with care taken to avoid pipetting up larger tungsten particles at the bottom of the solution. The assemblies were carefully moved to a dessicator containing Drierite™ crystals. The dessicator was stored in a dark cabinet and the macrocarriers were allowed to dry completely. The final result is a macrocarrier assembly with a layer of dried fine tungsten particles coated with DiI stain. These disks were used within 24 hours to prevent any degradation of the DiI.

**DiOlistic Labeling Using BioRad Helios™ Chambered Gene-gun**

Four tissue sections were removed from storage in 25 mM PBS containing wells and placed on a culture dish. The tissue was manipulated using a soft fine-tipped paintbrush to flatten the tissue against the bottom of the culture dish. Excess PBS was removed from the dish using a disposable plastic pipette to avoid slowing or alteration of the trajectory of DiI-conjugated tungsten beads. Sections were placed 8.2 cm from the DiI-tungsten coated macrocarriers and fired upon at a pressure of 900 psi. Stained sections were removed and placed into 24-well plates containing fresh 25 mM PBS and allowed to incubate at room temperature for 24 hours to allow adequate diffusion of DiI along neurites. Tissue sections were wet-mounted onto standard microscope slides for imaging within three days. Slides were stored at 4°C on damp towels to prevent evaporation of PBS from wet mounts.
Confocal Microscopy and 3D Modeling

Labeled tissues were imaged using a confocal microscope after the 24-hour incubation period. DiI fluoresces at the same excitation wavelength as TRITC. The TRITC setting was chosen as the preset is widely available across fluorescence microscopy programs. LASX software was used to acquire and save image sequences. Neurons were chosen based on their distinguishability from surrounding neurons and the clarity of projections. Neurons used for dendritic spine analysis required higher resolution to be selected for imaging in order to achieve accurate spine counts. The slides were placed on the microscope and manually focused using the fine focus knob. The depth of field is then adjusted using the LASX software to select a “start” and “end” point that includes the entirety of the neuron along the Z axis. Serial images were then taken with the number of images depending on the type of analysis for which the neuron was chosen. Neurons selected for dendritic spine analysis were imaged at an increased size of 1024x1024 and an increased number of serial images ranging from 50-70 images per neuron. These images would eventually be virtually compiled to generate a 3D z-stack model using the free software ImageJ.

Dendritic Spine Analysis

Confocal image sequences were imported to ImageJ and prepared for use with the Simple Neurite Tracer plug-in (available in the free ImageJ toolkit, FIJI). The images were split into RGB color channels and only the red channel was used for spine analysis. The scale was manually set using the “set scale” function in ImageJ and the scale bar saved onto all images of the stack when obtained using the LASX imaging software.
The Simple Neurite Tracer generates a tracing of the neurites by searching for a path of contiguous pixels between two points manually selected along a neurite. Each dendrite of the neurons imaged for spine analysis was traced from the soma to its terminal end. The dimensions of the tracings are displayed along the path log and were recorded to determine spine density. The spines from each dendrite were counted manually and divided by the length of each dendrite to determine spine density.

**Quantifying Dendritic Arborization using Sholl Analysis**

Confocal image sequences were imported to ImageJ and again prepared for use with the Simple Neurite Tracer plug-in. The neurites were traced along their entirety from a shared point in the center of the soma. 3D stacks were made alongside image sequences to allow for visualization of the neuron as a single model. This 3D model was manipulated to more easily visualize the origin of each neurite being traced. The result is a group of tracings that includes each dendrite and its branches starting from a single point of origin in the center of the soma. The axons were distinguished by medial positioning, presence of a tapered axon hillock, and absence of spines along the neurite. Axons were not included in the calculation of dendritic arborization. The tracing was then used for Sholl analysis using the Sholl analysis function within the Simple Neurite Tracer plug-in. Three neurons were selected for sample Sholl analysis, two experimental and one control. These neurons were selected due to their low background interference and the desired staining of the entire length of the respective dendrites.

**RESULTS**

**Optimization of DiOlistic Staining Procedure using Helios Chambered Gene Gun**
Traditional preparation of macrocarriers involves the use of the binding agent polyvinylpyrrolidone. The inclusion of this polymer assists in the adherence of tungsten-DiI microcarriers to the sides of sections of tubing serving as macrocarriers. This agent was unnecessary in the preparation of the macrocarrier disks specialized for use in chambered gene guns. Furthermore, the increased adherence provided by polyvinylpyrrolidone actually caused the solution to dry into a solid on the surface of the macrocarrier disk instead of the desired coating of tungsten-DiI microcarriers. This solid conglomerate was fixed to the macrocarrier disk and, thus, would not dissociate from the disk upon firing. Decreased concentrations of polyvinylpyrrolidone in solution were trialed with the same result. Omission of this agent from the solution allowed sufficient adherence to the disk and appropriate dissociation from the disk during the firing process. For this reason, a solution consisting of only DiI-coated tungsten beads suspended in ddH₂O was selected for preparation of macrocarrier disks.

The next parameters examined were the distance between tissue sections and the macrocarrier disks during the firing process and the number of sections stained during a single firing session. It was hypothesized that too great of a distance would result in insufficient penetration of tungsten microcarriers into tissue sections while distances that were too short would cause over-penetration and a higher concentration of fluorescence in centrally located tissues. This increased fluorescence would potentially cause overstaining of the tissues towards the center of the culture plate. The organization of the gene gun itself allows a limited number of distances between the macrocarrier disk and the tissue below. Two slots were chosen for this procedure, the closer slot placing the tissue at a distance of 5.1 cm from the macrocarrier and the further slot placing the tissue
at a distance of 8.2 cm from the macrocarrier. As shown in Table 1, a distance of 5.1 cm between the tissue and macrocarrier was not viable for producing quantifiable neurons. The tissue contained excessive amounts of fluorescent dye, particularly in the areas of tissue located nearest the center of the plate. The quantifiable neurons observed in the trials staining four tissue sections at a distance of 5.1 cm were located in the areas of tissue located furthest from the center of the plate. Although the degree of overstaining in single section staining trials was improved at a distance of 8.2 cm, the overstaining was still sufficient to render the tissue unusable. This is likely due to the central placement of the DiI-conjugated tungsten particles on the macrocarrier disk resulting in the majority of particles being fired into the center of the plate. The degree of overstaining was markedly decreased in multiple section staining trials at a distance of 8.2 cm. The arrangement of the tissues placed each tissue further from the center of the plate. This, combined with the increased distance that the microcarriers traveled before striking the tissue, allowed for the appropriate distribution and tissue penetration of the particles. The protocol used for the remainder of this study used the arrangement of four tissue sections per shot to allow for appropriate staining while decreasing the total cost for producing each quantifiable neuron.

Dendritic Spine Analysis of Pyramidal Neurons in the Motor Cortex

The DiOlistic labeling procedure was effective at generating high-resolution images of dendrites and their associated spines. These spines were distinguishable from neighboring spines and sufficient for manual counting (Figure 4). Using the dimensional data from the tracings obtained using Simple Neurite Tracer, the density was calculated by dividing the total dendritic spine count by the total dendritic length (Table 2).
Interestingly, the total number of dendrites measured in the 5-HT treatment group was twice as high as the control group (24 vs 11, respectively). The total dendritic length of the treatment group was only greater than the control group by a factor of 1.7 (7290 µm vs 4241 µm, respectively). Total dendritic spine density was greater in the 5-HT treatment group. The preliminary data generated using this procedure was not of high enough power to generate statistical significance, however.

**Quantification of Motor Cortex Pyramidal Neuron Dendritic Arborization using Sholl Analysis**

Sholl analysis employs the use of three-dimensional concentric “shells” beginning at the neuronal body and are incrementally placed radially surrounding the neuron. Each point at which a dendrite and its corresponding branches crosses a shell is recorded as an intersection. The number of intersections with these shells can be used to infer the degree of branching of that dendrite, or the arborization of that dendrite. The cumulative arborizations of the dendrites from a single neuron can be viewed as the degree of branching of the neuron as a whole. The connective potential of each neuron can be extrapolated from the arborization of this neuron. The mean intersections of the dendrites of each neuron can be compared to the dendrites of another to essentially determine which neuron has the greater ability to interact with nearby neurons. The Sholl analysis data of the three neurons selected is shown in Figures 5, 6, and 7.
CONCLUSION AND FUTURE DIRECTIONS

DiOlistic labeling using the Helios® chambered gene gun was successful in producing images of high enough quality for statistical analysis. The preparation of disks without the binding agent polyvinylpyrridone produced an even spread of DiI-tungsten beads that would dissociate from the disk and penetrate tissue samples at a firing pressure of 900 psi. This was found to be most effective at a distance of 8.2 cm. At this distance, tissue samples were penetrated by an adequate amount of DiI without overstaining. The even distribution of DiI allowed for the placement of multiple tissue sections on each slide without compromising the degree of staining, further reducing material costs. Using Simple Neurite Tracer, the dimensions of these projections was calculated in three-dimensional space. The staining method was able to stain neuronal projections from the connection to the soma to the point of termination. This allowed for Sholl analysis to accurately include each dendrite and its branches, producing data on the potential connectivity of each neuron represented as the mean intersections of that neuron. This method allowed for a sufficient resolution to manually count dendritic spines along the length of dendritic projections. Unfortunately, several automatic counting methods were not able to accurately count the number of spines. This does present an inherent issue with observer bias and the observed number of spines is assumed to be lower than the actual count due to spine placement along the z axis. Previous automated methods tend to show the same bias, however. The sample sizes used in the preliminary data collection step was not adequate to produce statistical significance. The ability to produce results was achieved, however, and allows for use of this method in the future to analyze the difference in neuronal morphology between control and 5-HT treatment animals.
The dendritic arborization of cortical neurons can be examined using the DiOlistic staining procedure and the free program ImageJ. With a larger sample size, a statistical difference between the two groups can be determined. A statistically significant difference between motor cortical neurons from control groups versus those from 5-HT treatment groups would provide information towards the fine motor deficits observed in ASD patients. A decrease in overall dendritic arborization in 5-HT treatment groups would suggest that these neurons are less capable of synapsing with nearby neurons and could potentially point to an underperformance of the motor cortex to plan and initiate fine movement. Furthermore, the dendritic spine density of these neurons can be calculated to further analyze this connectivity. A decrease in dendritic spine density in the 5-HT treatment group would suggest a lower number of synaptic terminals shared with surrounding neurons.
REFERENCES


Figure 1: Experimental timeline. This flowchart shows the treatments received and the dates at which treatments were delivered to control and treatment rats.
Figure 2: Tissue arrangement & gene gun assembly for DiOlistic labeling. (A.) Illustration of tissue section orientation for labeling by DiOlistic transfection. Tissue was arranged as shown in groups of: 1, 3, or 4 section(s). (B.) Illustration showing the altered distances of tissue sections from the macrocarrier disk and projected spread of DiI-conjugated tungsten beads (shown in red).
Figure 3: Confocal imaging of DiOlistically labeled neurons. (A.) Illustration showing the area of the rat motor cortex. (B.) Example of a quantifiable neuron obscured by gross overstaining. (C.) Neuron labeled with DiI with an anterograde direction of diffusion. The complete staining of the somatic membrane suggests that the DiI entered in the neuronal soma and progressed radially along the neuronal projections. (D.) Neuron labeled in a retrograde direction of diffusion. The increased level of fluorescence in the apical dendrite and incomplete staining of the soma suggests that the DiI entered the dendrite and progressed towards the soma and along the other projections.
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**Table 1: Results of DiOlistic Staining Optimization Trials.** Brains used in this trial were harvested from breeder rats not involved in this study.
Figure 3: Examples of Pyramidal Neuron Tracings using Simple Neurite Tracer. Images above were changed to grayscale by splitting color channels and using only the red channel. The pink overlays are the tracings generated by the Simple Neurite Tracer plug-in. These tracings were used to calculate dendritic length for spine density analysis as well as performing the Sholl analysis for quantifying dendritic arborization.
Table 2: Dendritic Spine Density Analysis.
The length of dendrites was calculated using the Simple Neurite Tracer plug-in. Dendrites were counted manually. Dendritic spine density was calculated by dividing the total number of dendritic spines by the total dendrite length.
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