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Assessment of Motor Function, Motor Learning, & Olivary Climbing Fiber Distribution within Developmental Hyperserotonemia Rat Model for Autism Spectrum Disorder

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**ASSESSMENT OF MOTOR FUNCTION, MOTOR LEARNING, & OLIVARY
CLIMBING FIBER DISTRIBUTION WITHIN DEVELOPMENTAL
HYPERSEROTONEMIA RAT MODEL FOR AUTISM SPECTRUM DISORDER**

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

Elizabeth Diane Holland

May 2019

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CLIMBING FIBER DISTRIBUTION WITHIN DEVELOPMENTAL
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Biomedical Sciences

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Elizabeth Diane Holland

ABSTRACT

While Autism Spectrum Disorder (ASD) is defined by deficits in social communication, compromised motor function and motor learning have been increasingly reported. Motor deficits could compound social impairment through delayed language acquisition, reduced opportunity for social interaction, and affected nonverbal communication. One area of interest in the investigation of motor dysfunction is the cerebellum, where altered cerebellar structure and connectivity have been reported in those diagnosed with ASD. Morphological and functional changes in cerebellar circuitry could disrupt motor skill development and may be associated with developmental alterations of the serotonergic system. Elevated blood serotonin in perinatal development, developmental hyperserotonemia (DHS), is the most consistent neurochemical finding reported in ASD and has been implicated in the pathogenesis of the disorder. The present investigation examined the link between DHS, cerebellar development, motor function, and motor learning in Sprague Dawley rats. Motor learning of DHS animals was assessed through repetitive balance beam motor training and testing, the extent of improvement throughout trials being reflective of motor learning and potential motor skill rescue. Investigation of cerebellar circuitry was performed with immunohistochemical labeling of cerebellar Purkinje cells (PCs) with anti-calbindin and olivary climbing fibers with anti-vesicular glutamate transporter 2 (VGlut2), then assessed using confocal microscopy and ImageJ particle analysis.

KEYWORDS: Autism Spectrum Disorder (ASD), developmental hyperserotonemia (DHS), motor control, motor learning, cerebellum, Purkinje cells (PCs), olivary climbing fibers

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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

Neurodevelopmental disorder Autism Spectrum Disorder (ASD) is characterized by deficits in social communication and interactions with concurrent presentation of restrictive, repetitive patterns of behavior (APA, 2013). The population of individuals with ASD, however, is heterogeneous with diagnostically defined manifestations varying in presentation, frequency, and severity. Additionally, the majority of those with ASD experience comorbidity of other diagnoses and symptoms, one of the most common being compromised motor control (Lai, Lombardo, & Baron-Cohen, 2014). More than 79% of those with ASD experience a motor deficit of some kind (Lai et al., 2014). This has resulted in some experts advocating for the addition of motor deficit to current diagnostic criteria (Autistic Self Advocacy Network, 2012; Jeste, 2011). The inability to maintain appropriate motor control could compound social impairment innate to the disorder through a variety of ways including: decreased opportunity for social interactions (Focaroli, Taffoni, Parsons, Keller, & Iverson, 2016; Iverson, 2010; Leonard, Elsabbagh, & Hill, 2014; Nickel, Thatcher, Keller, Wozniak, & Iverson, 2013; Sipes, Matson, & Horovitz, 2011; Travers, Powell, Klinger, & Klinger, 2013), delayed or compromised language development (Focaroli et al., 2016; Iverson, 2010; Leonard et al., 2014; Mostofsky, Goldberg, Landa, & Denckla, 2000; Nickel et al., 2013), and/or impaired nonverbal communication orchestrated by fine motor movements of facial muscles (Cook, Blakemore, & Press, 2013; Loveland et al., 1994; Murias et al., 2017).

Individuals with ASD also display variable severity of motor learning deficit. Investigation of explicit motor learning, learning generating verbal knowledge of improved motor performance, has resulted in conflicting reports (Kleynen et al., 2014). Some have

detected intact explicit motor learning (Watanabe, Ikeda, & Miyao, 2010) while others have found it disrupted, the participants unable to verbally express recognition of patterns in a button pushing task (Izadi-Najafabadi, Mirzakhani-Araghi, Miri-Lavasani, Nejati, & Pashazadeh-Azari, 2015). It is possible communicative deficits innate to the disorder may confound results in the study of explicit motor learning within populations affected by ASD. Similarly, investigation of implicit motor learning, learning involving minimal to no verbal knowledge of improved movement performance, has resulted in variable observations (Kleynen et al., 2014). Some researchers have reported intact implicit motor learning in the ASD population (Brown, Aczel, Jiménez, Kaufman, & Grant, 2010) while others have found it compromised, observing a reduction of corrective adjustments on motor tasks with repeated exposure (Izadi-Najafabadi et al., 2015; Marko et al., 2015; Mostofsky et al., 2000; Travers, Kana, Klinger, Klein, & Klinger, 2015). However, with increasing reports of compromised implicit motor learning, it is possible contradictory findings are related to confounding factors such as not controlling for cognitive impairment across participants. Implicit motor learning testing in individuals with ASD may provide more accurate results by the inherent removal of the communicative component of assessment.

In investigations of motor dysfunction within populations affected by ASD, disrupted or altered cerebellar circuitry is frequently proposed as a potential cause for reduced motor coordination (Cook et al., 2013; Fatemi et al., 2012; Hollander, Wang, Braun, & Marsh, 2009; Lai et al., 2014; Laidi et al., 2017; Mosconi, Wang, Schmitt, Tsai, & Sweeney, 2015; Mostofsky et al., 2009, 2000). The cerebellum is associated with refinement and coordination of motor movements and contributes to regulation of motor learning through correction of erroneous signals, allowing improvement on motor tasks. Cerebellar structure, cerebellar cell populations,

and cerebellar connectively are commonly altered in patients with ASD (Fatemi et al., 2012; Hoxha et al., 2017; Mosconi et al., 2015; Mostofsky et al., 2009). Morphological and functional changes in cerebellar circuitry associated with developmental alterations of the serotonergic system could disrupt development of motor control and motor learning (Adamsen et al., 2014; Alzghoul et al., 2012; Kane et al., 2012; Mostofsky et al., 2000; Sprowles et al., 2016). Increased circulating serotonin is the most consistent neurochemical finding within individuals with ASD, approximately one third of individuals showing a 40-70% increase of platelet bound serotonin (Hough & Segal, 2016; Whitaker-Azmitia, 2005). Serotonin contributes to postnatal development of the cerebellum, strengthening the argument of this developmental factors involvement (Purves et al., 2001). The present investigation examined relationships between development of motor control and motor learning, cerebellar circuitry, influence of the serotonergic system, and their relevance to ASD.

LITERATURE REVIEW

Autism Spectrum Disorder

ASD is increasingly prevalent. Latest reports approximate 1 in 59 children within the United States receive an ASD diagnosis, a 15% increase from estimates released in 2012 (Baio et al., 2018). However, it is not definitive that this truly reflects increased incidence. Alterations to diagnostic criteria or greater community awareness of this disorder may contribute to higher rates of identification. ASD transcends racial, ethnic, and socioeconomic groups and is known to display a sex bias with approximately 4 males diagnosed for every 1 female (Baio et al., 2018). Additionally, little is known regarding ASD etiology and while experts continue working towards development of biological diagnostic markers, accurate tracking of prevalence remains challenging. However, increased presentation of this disorder compounded by its heterogeneous nature and broad scope of affected individuals creates an intriguing topic of research.

Diagnosis of ASD is contingent on adherence to criteria described within the current Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) (Figure 1). Manifestations of deficits in social communication and restrictive, repetitive patterns of behavior must be visible in early childhood, generally prior to eight years of age (APA, 2013). However, variability of presentation is observed within directly associated core symptoms and those beyond explicit mention within the DSM-5, such as the commonly reported deficit in motor function (Lai et al., 2014).

Social impairment may encompass different aspects of communication in a broad range of severity; however, deficits in all three criteria are required for ASD diagnosis (APA, 2013). One area of communication affected in individuals with ASD is social-emotional reciprocity. A

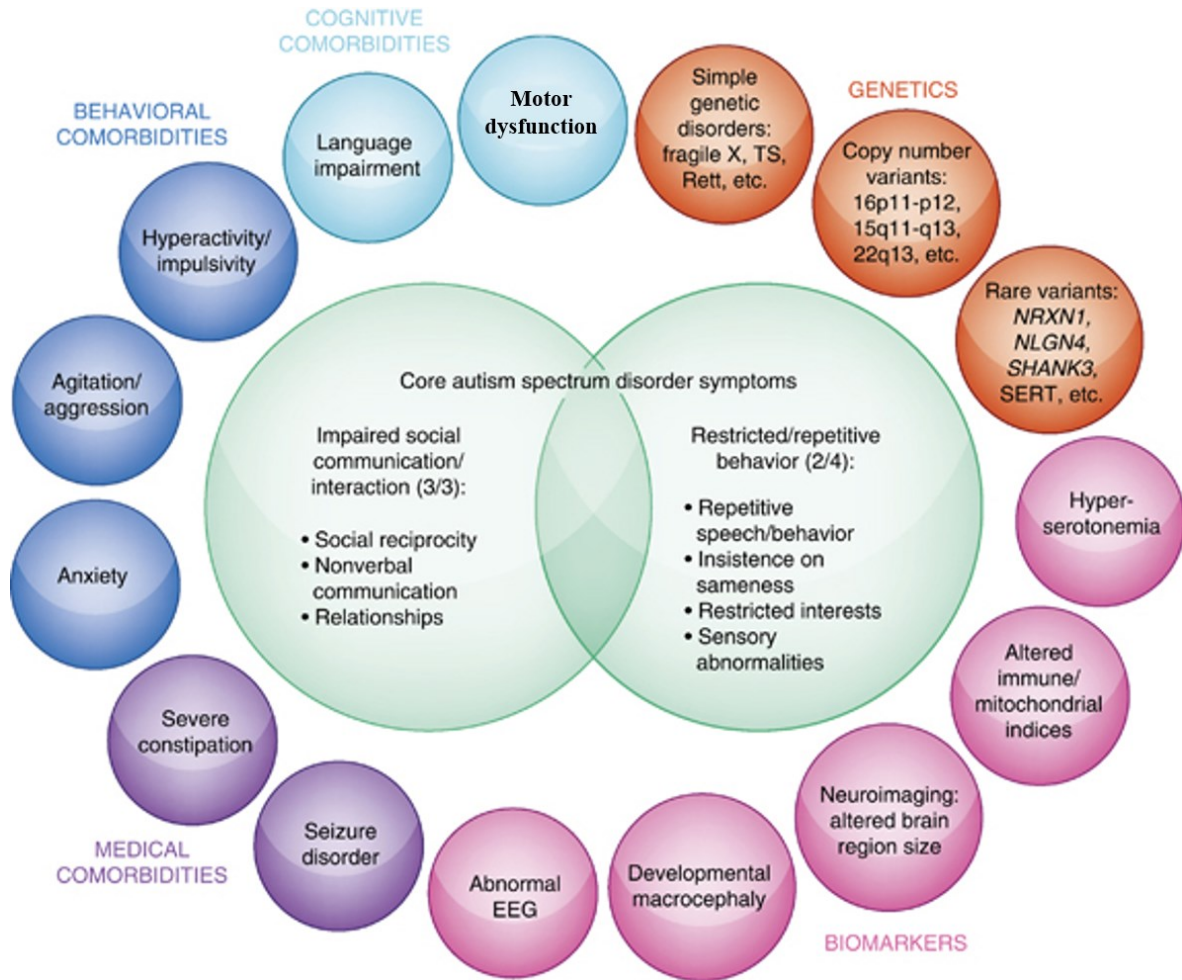


Figure 1: Diagnostic Criteria of Autism Spectrum Disorder with Associated Comorbidities. Illustrative representation of core diagnostic criteria of ASD, per the current DSM-5, with comorbidities, genetics, and biomarkers frequently associated with the disorder.

few examples of possible presentation include limited or inappropriate: initiation of social interactions, reactions to social approach of others, and/or sharing of emotions or common interests. The second criterion of impaired sociability involves nonverbal and verbal communication, manifesting as reduced or absent eye contact, understanding of gestures and facial expressions, and/or comprehension of appropriate body positioning. Symptoms associated with postural stability, inappropriate body positioning, or motor coordination, fine motor control being required for subtle eye movements necessary for eye contact, suggest reciprocity regarding development of social communication and motor development. In severe presentations of ASD the individual may display scripted speech, manifesting as limited responses available regardless of novel situations, inappropriate tone or volume when speaking, delayed speech acquisition, or the absence of verbal expression in its entirety. Additionally, motor development is associated with the development of language skills, due to a myriad of reasons discussed in later sections (Iverson, 2010). Therefore, the relationship between development of motor control and nonverbal and/or verbal communication appears robust. The final criterion regarding deficit in social communication includes compromised development, maintenance, and/or understanding of relationships. This may be reflected by inability to understand social context, the concept that one responds in accordance to environmental and situational factors. There may also be a reduction or absence of interest to interact with peers or difficulty engaging in imaginative play (APA, 2013).

Restrictive, repetitive patterns of behavior are also variable in severity and presentation, with two of the four presentations required to receive an ASD diagnosis (APA, 2013).

Restrictive, repetitive patterns of behavior potentially displayed are use of stereotyped or repetitive speech, motor movements, or object use. Examples of these behaviors include:

pronoun reversal, in which individuals use “you” in the place of “I,” stereotyped body movements such as rocking back and forth, swaying, or spinning, and the repetitive use of objects such as continuously opening and closing doors. Stereotyped movements have been proposed to be a maladaptive compensatory mechanism representative of disordered motor control at the response-selection phase of motor planning, selecting the appropriate motor response to external stimuli, or execution of said motor response (Ravizza, Solomon, Ivry, & Carter, 2013). Another indication of restrictive, repetitive patterns of behavior is rigidity associated with routine adherence and resistance to change. Expression of restricted, fixed interests is the third possible criterion under this category. A narrow range of interest, preoccupation with select objects, topics or activities, and being perfectionistic are examples of this behavior. The final possible manifestation of restrictive, repetitive behaviors includes alterations in sensitivity to sensory cues, with either increased or decreased sensitivity. The affected individual may have a unique attraction or aversion to specific experiences in all domains of sensory stimuli. Sensorimotor processing appears altered within the affected population when compared to typically developing individuals. This effect has been reported when affected and unaffected participants experience the “rubber hand illusion” where subjects visually observe a rubber hand being touched while their real hand is out of view and is synchronously stimulated. This can invoke the illusion of experiencing stimuli applied to the rubber hand on the real hand, even when the stimuli is no longer being applied to the participants’ hand. (Paton, Hohwy, & Enticott, 2012). Sensorimotor processing is critical for appropriate movement adjustments in response to external stimuli; thus, if this is altered in those with ASD, motor coordination may also be affected. Therefore, development and integration of the motor system appears pertinent to another core feature of ASD.

Motor Function & Autism Spectrum Disorder

As previously described, motor function is one of the most consistent comorbidities displayed by individuals with ASD, observed in over 79% of the affected population (Lai et al., 2014). Recommendation for motor deficits addition to DMS-5 diagnostic criteria is partially due to this robust statistical data (Autistic Self Advocacy Network, 2012; Jeste, 2011; Lai et al., 2014). However, motor abnormalities are also easily observed and quantified. Alterations in sensorimotor processing, for example, may be difficult to objectively detect and assess. Therefore, motor dysfunction as diagnosis criteria could potentially increase diagnostic accuracy without increased chance of misdiagnosis (Autistic Self Advocacy Network, 2012). Additionally, it is suggested that features of some core diagnostic criteria, such as stereotyped movements, are reflective of motor deficit (Leekam, Prior, & Uljarevic, 2011; Ming, Brimacombe, & Wagner, 2007; Ravizza et al., 2013). Therefore, compromised motor control may already contribute to formal ASD diagnosis.

Motor control is defined as the process of cognitively activating various brain regions and coordinating muscle movements to complete a desired task (Ito, 2000). This can be further subdivided into gross motor control and fine motor control. Gross motor control refers to modulation of large movements involving the upper and lower limbs, in which compromised function could alter gait, postural stability, and overall coordination. Gross motor control deficits manifesting as decreased motor coordination, abnormal arm movements, altered gait, and postural instability are commonly observed across all ages of individuals with ASD, unlike typically developing individuals in which postural stability improves throughout childhood (Behere, Shahani, Noggle, & Dean, 2012; Bhat, Landa, & Galloway, 2011; Fournier, Hass, Naik, Lodha, & Cauraugh, 2010; Minshew, Sung, Jones, & Furman, 2004; Nickel et al., 2013; Rinehart

et al., 2006; Staples & Reid, 2010; Travers et al., 2013). Due to the pervasive nature of gross motor coordination deficit and its reliable detection as early as 1-2 years of age, it has been proposed to be an early indicator of ASD (Bhat et al., 2011; Leonard et al., 2014; Nickel et al., 2013).

Differing from gross motor control, fine motor control refers to small, precise movements associated with dexterity, subtle facial expressions, and modest mouth and eye adjustments. Fine motor control is commonly affected in individuals with ASD (Bhat et al., 2011; Focaroli et al., 2016; Hilton, Zhang, Whilte, Klohr, & Constantino, 2012; John et al., 2016; Leonard et al., 2014; Mari, Castiello, Marks, Marraffa, & Prior, 2003; Sipes et al., 2011). This can be observed within ASD populations as compromised “reach-to-grasp” development, individuals displaying decreased dexterity and movement planning when reaching out to grasp small objects, requiring coordination of various digits (Mari et al., 2003). This phenomenon may also be observed as difficulty stacking blocks, a task requiring delicate coordination for approach, placement of hands, and positioning of one block onto the other (Focaroli et al., 2016). Deficits of fine motor control may be more sensitive to detection at an earlier age. There are reports of compromised fine motor function in infants assessed using the Mullen Scales of Early Learning (MSEL) standardized motor test, primarily observing grasping behavior as fine motor assessment, as early as 6-7 months of age in at-risk populations (Bhat et al., 2011). Children are considered at-risk for a diagnosis of ASD if they possess an affected immediate family member, traditionally a sibling (Bhat et al., 2011; Leonard et al., 2014). Other researchers, however, have reported that detection of compromised fine motor control is not reliable prior to one year of age (Landa, Gross, Stuart, & Bauman, 2012; Libertus, Sheperd, Ross, & Landa, 2014).

Alterations in motor control development appear to have implications regarding development of communicative abilities. There is a relationship between motor control performance and socially relevant tasks such as locomotion to seek out social interactions, acquisition of verbal skills, and development of nonverbal expression (Dziuk et al., 2007; Focaroli et al., 2016; Fournier et al., 2010; Hilton et al., 2012; Iverson, 2010; Leonard et al., 2014; Mostofsky et al., 2000; Nickel et al., 2013; Sipes et al., 2011; Travers et al., 2013). Deficient motor skills could compromise any of the aforementioned domains, compounding communicative deficits, symptoms innate to ASD. Reduced social behavior, characteristic to ASD, does not appear to be present at birth, instead presenting after a developmental regression at approximately 6 months of age (Landa et al., 2012; Ozonoff et al., 2010). Coincidentally, studies show similar time frames for reliable detection of deficits in motor coordination (Bhat et al., 2011). Such is increasingly suggestive that delayed or deficient motor skills could participate to emergence of reduced sociability.

Deficits in gross motor control, manifested as postural instability, may result in delayed onset of crawling, exacerbating avoidance of social interactions through decreased opportunity (Focaroli et al., 2016; Iverson, 2010; Leonard et al., 2014; Nickel et al., 2013). Additionally, postural instability severity has been correlated with severity of stereotyped, repetitive behaviors. Those with more severe stereotyped, repetitive behaviors display less postural symmetry and more postural wavering when performing balance related tasks, like standing on one foot with one's eyes closed (Travers et al., 2013). This further illustrates possible concurrent development of motor control and other processes associated with diagnostic criteria (Travers et al., 2013). Postural instability may also generate feelings of social isolation throughout childhood of affected populations through the inability to cater to social demands such climbing a tree or

riding a bicycle (Travers et al., 2013). This could discourage desire to engage with peers, increasing aversion to social interactions and environments.

Prevalence of motor deficit is frequently associated with compromised language abilities, reflected by delayed speech acquisition and reduced vocabulary (Focaroli et al., 2016; Iverson, 2010; Leonard et al., 2014). The ability to support oneself in an independent, unsupported sitting position is implicated in the development of consonant-vowel vocalizations; frequency and control over such vocalizations increase when infants begin to sit independently (Iverson, 2010). Additionally, decreased efficiency in creating subtle mouth movements vital for vocalization may effect language development as well as infantile object exploration (Iverson, 2010).

Compromised fine motor skills could inhibit one's ability to follow caregivers' gaze or make eye contact if one is in motion, impeding bonding between the infant and guardian (Murias et al., 2017). Additionally, eye contact is associated with emotional reciprocity, where eye contact is usually followed by a social smile as a means to nonverbally acknowledge attention (Laidi et al., 2017). Oculomotor control is vital for appropriate sensory integration which when compromised could exacerbate motor dysfunction. Studies have also reported affected facial mirroring. When affected participants are exposed to representative images of others expressing an identifiable emotion, they themselves display a distorted imitation (Loveland et al., 1994). This could be illustrative of altered sensory integration or perception, where the affected individual has difficulty understanding the emotion being nonverbally represented (Cook et al., 2013). Conversely, this could display compromised fine motor skills altering an individual with ASD's physical capabilities to perform emotional facial mirroring (Cook et al., 2013). Ultimately, a deficit in motor coordination, whether in the gross or fine motor domain, could compound impaired communicative abilities within populations affected with ASD.

Motor Learning & Autism Spectrum Disorder

With the majority of individuals with ASD presenting compromised motor control, there has been investigation regarding abilities to improve on motor tasks. Motor learning refers to the process in which one acquires functional motor skill (Gentile, 1998). In this skill procurement, motor learning can manifest as increased efficiency or reduced errors upon repeatedly completing a motor task; this improvement reflects alterations of neuronal connectivity in various brain regions (Izadi-Najafabadi et al., 2015; Yang & Li, 2012). Motor learning can be subdivided into two distinct processes, each suspected to activate different brain networks: explicit motor learning and implicit motor learning (Kleynen et al., 2015; Yang & Li, 2012).

Explicit motor learning refers to learning generating verbal knowledge of movement performance, involving cognitive stages within the learning process dependent on working memory involvement (Kleynen et al., 2014). Accordingly, explicit motor learning is declarative, with conscious memory of facts and events that can be recalled at will. When assessing explicit motor learning within an experimental setting, it is crucial to verbally inform the participant of the nature and inherent goal of the task, so that the individual may explain it to the researcher upon learning occurring (Izadi-Najafabadi et al., 2015; Kleynen et al., 2015). Understandably, when working with populations of individuals with compromised communicative abilities, such as those with ASD, verbally expressing explicit motor learning may remain challenging. Such difficulties, compounded by varying level of cognitive impairment across groups, may contribute to conflicting findings. Some researchers have reported intact explicit motor learning within individuals with ASD (Watanabe et al., 2010), however, others have reported that there is an apparent explicit motor learning deficit (Izadi-Najafabadi et al., 2015). These discrepancies

ultimately require more research prior to making conclusive statements regarding explicit motor learning in individuals with ASD.

Implicit learning is defined as learning that progresses with no or minimal increase in verbal knowledge of movement performance, without conscious awareness (Kleynen et al., 2014). Implicit learning is, therefore, procedural. The individual learns how to accomplish a task and does not consciously recognize learning, but rather demonstrates it. Differing from explicit motor learning, when assessing implicit motor learning specific instructions provided to the participants should be limited or avoided entirely (Kleynen et al., 2015). Research of implicit motor learning within individuals with ASD remains mixed in which, again, some researcher report intact implicit motor learning (Brown et al., 2010), while others have found implicit motor learning to be compromised within the affected population (Izadi-Najafabadi et al., 2015; Marko et al., 2015; Mostofsky et al., 2000; Travers et al., 2015). However, there is still concern regarding studies that did not control for cognitive impairment across testing groups, a potential source of contradictory findings.

In the examination of motor learning, it has been proposed that individuals with ASD primarily learn from proprioceptive information as opposed to visual sensory cues employed by neurotypical individuals. This allows implicit motor learning to occur, but in a slower, less accurate fashion (Haswell, Izawa, Dowell, Mostofsky, & Shadmehr, 2009; Paton et al., 2012). The ability to successfully adjust motor movement in response to integrated visual and proprioceptive information is critical for motor learning (Marko et al., 2015). Studies involving clinical intervention with implementation of motor training have observed improved balance and/or motor coordination within the affected ASD population (Travers et al., 2018). This illustrates that motor learning is possible, perhaps merely compromised, within individuals with

ASD. Longitudinal studies investigating long-term outcomes after motor skill training are necessary to assess whether early training regimens could lessen or alleviate deficits in motor control and associated communicative consequences.

The Cerebellum & Autism Spectrum Disorder

A multitude of neuronal structures may be implicated regarding compromised motor function and motor learning within populations of those affected by ASD, however, aberrant cerebellar connectivity or altered morphology are commonly suggested (Cook et al., 2013; Fatemi et al., 2012; Hollander et al., 2009; Jeste, 2011; Lai et al., 2014; Laidi et al., 2017; Mosconi et al., 2015; Mostofsky et al., 2009, 2000; Zeeuw & Brinke, 1990). The cerebellum is a region of the brain within vertebrates that is associated with regulation and refinement of motor movements. This can be reflected with management of posture, balance, and generation of smooth, coordinated actions. This region is also associated with procedural motor learning, particularly detection and correction of erroneous motor signaling, allowing for improvement in speed or error rate on motor tasks (Piochon et al., 2014; Zeeuw & Brinke, 1990). The correlation of cerebellar structures and ASD is due to functional significance of the region and studies involving patients with ASD.

Typically developing cerebellum are composed of three distinct layers (Figure 2). The most superficial, molecular layer, is composed of inhibitory stellate cells and basket cells. This layer also contains Purkinje cell (PC) dendritic arbors and axonal projections of granule cells, parallel fibers. The next layer, moving deeper into the cerebellar cortex, is the PC layer which contains large PC bodies. The deepest layer, the granule cell layer, is densely packed with granule cells. The granule cell layer also contains inhibitory Golgi cells, PC axons, olivary

climbing fibers originating from the inferior olive of the medulla oblongata, and mossy fibers originating from the lateral reticular nucleus and pontine nuclei (Purves et al., 2001).

Regulation and refinement of motor movement occurs through delicate balance of excitatory and inhibitory signaling associated with the various layers and cell populations of the cerebellar cortex (Figure 2 & Figure 3). GABAergic, inhibitory PCs receive excitatory input from two cellular sources: one olivary climbing fiber, providing direct excitation, and many parallel fibers, providing indirect excitation, within the molecular layer (Purves et al., 2001). For this excitatory, glutamatergic signaling, neurons within the lateral reticular nucleus and pontine nuclei must be excited. The axonal projections of the aforementioned cell types, mossy fibers, then excite granule cells within the granule cell layer. The many specialized axonal projections of granule cells, parallel fibers, synapse onto elaborate dendritic arbors of PCs, allowing glutamatergic excitation. The other source of excitatory signaling originates from specialized axonal projections of the inferior olive, climbing fibers. One, singular climbing fiber will project excitatory signals onto the dendritic tree and soma of one PC (Figure 3) (Hoxha et al., 2017; Purves et al., 2001). However, the GABAergic PC cannot solely receive excitatory glutamatergic signaling, for it would then solely provide inhibition of motor movement to deep cerebellar nuclei. The regulatory nature of the cerebellar cortex is created by both excitatory and inhibitory signaling to PCs.

PCs receive direct inhibitory signaling from stellate cell and basket cell populations within the molecular layer and indirect inhibition from Golgi cell populations within the granule cell layer of the cerebellar cortex (Purves et al., 2001). Stellate cells receive excitatory signaling from parallel fibers; therefore, parallel fibers are capable of directly exciting PCs as well as indirectly inhibiting PCs through stellate cell excitation. Basket cells are excited by parallel

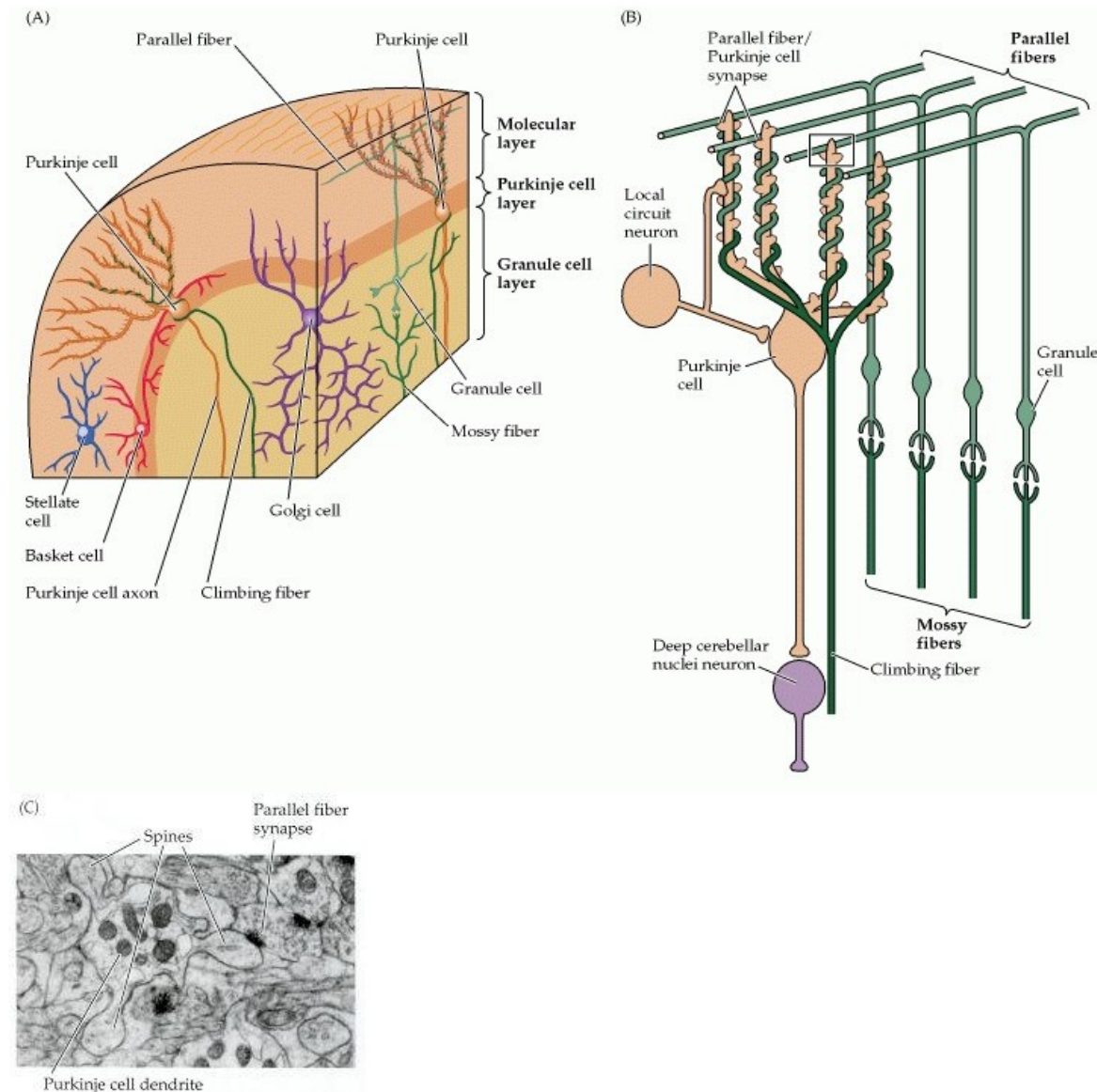


Figure 2: Neuronal Cell Populations and Circuitry of the Cerebellar Cortex. (A) Illustrative representation of the distinct cellular layers of the cerebellar cortex as well as associated neuronal cell populations. (B) Diagram illustrating convergent synaptic input onto Purkinje cell bodies and associated dendritic arbors from one climbing fiber and many parallel fibers. The boxed region is shown at a higher magnification within (C), an electron micrograph image showing three Purkinje cell dendritic spines receiving glutamatergic release, shown as dark electron dense regions, from three parallel fibers (image in C courtesy of A.-S. La Mantia and P. Pakic.). Copyright @ 2001, Sinauer Associates, Inc.

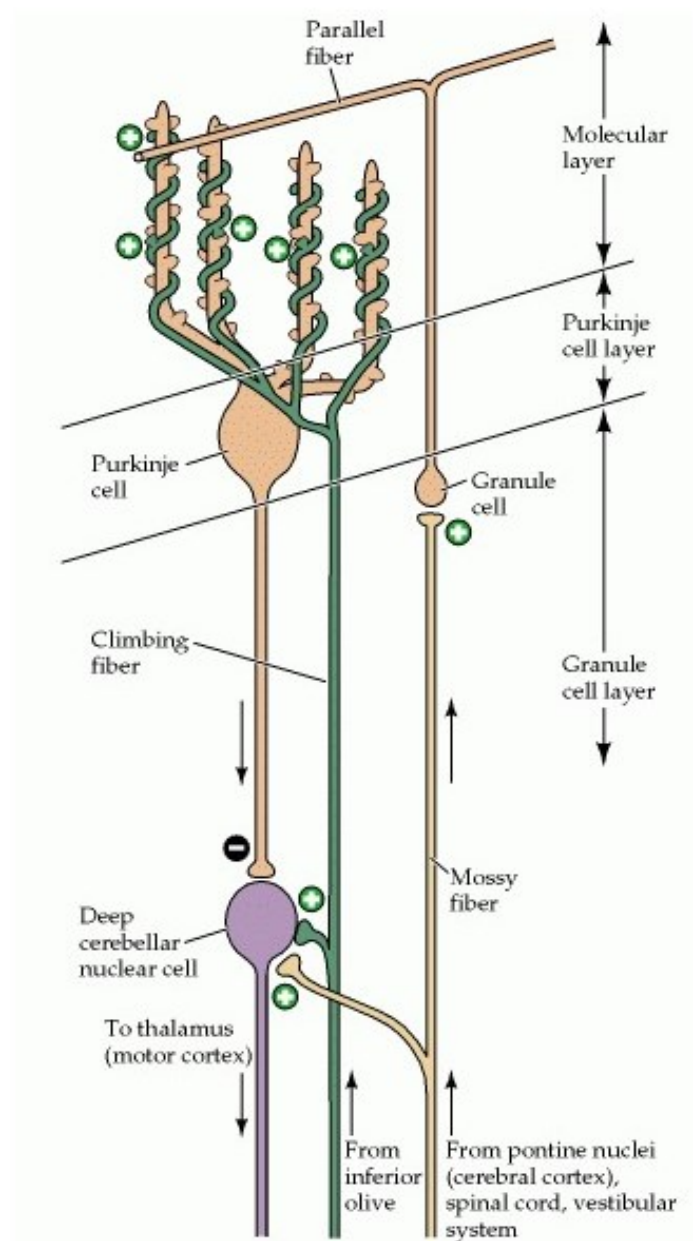


Figure 3: Purkinje Cell Connectivity, Modulation of Excitatory and Inhibitory Input.

Illustration of excitatory, glutamatergic, and inhibitory, GABAergic, synaptic connections onto Purkinje cells within various layers of the cerebellar cortex. Excitatory input is received by from one olivary climbing fiber and many parallel fibers, while inhibitory input is received from local circuit neurons: stellate cells, basket cells, and Golgi cells (not shown).

Additionally, one can see the inhibitory output of Purkinje cells to deep cerebellar nuclei.

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fibers, similarly to stellate cells, as well as olivary climbing fibers. Again, this displays duality of final output in which parallel fibers and olivary climbing fibers directly excite PCs, but indirectly inhibit the same cell population by excitation of inhibitory basket cells. Finally, PCs are indirectly inhibited by Golgi cell populations, with Golgi cells becoming excited by parallel fiber activation and consequently inhibiting the granule cells of which the parallel fibers originate (Purves et al., 2001). Coordination of this complex, seemingly redundant, neuronal network within the cerebellar cortex allows delicate fine-tuning of the summation of excitation the PCs receive and their ultimate inhibitory output to deep cerebellar nuclei.

The functional significance of appropriate PC modulation and associated activity within the cerebellar cortex is reflected within studies where such cell populations are disrupted. For example, mice with PCs deficient of calbindin, a calcium-binding protein critical for proper calcium response to excitation by parallel fiber and climbing fiber connectivity, are observed to experience deficits in motor coordination and in the processing of visual sensory cues (Barski et al., 2003). The selective knockout of *TSC1*, the gene coding for tuberous sclerosis complex 1 protein, within mouse PCs affects typical communicative skills within animals heterozygote for the gene mutation; mice homozygous for the mutation, however, additionally display motor deficit (Tsai et al., 2012). Mice with PCs devoid of PTEN, phosphatase and tensin homolog protein, display gross PC morphological disturbances, abnormal cerebellar signaling, and impairment in social communication and motor learning capabilities (Cupolillo et al., 2016). Additionally, disruption of *SHANK2*, the gene coding for PC postsynaptic scaffolding protein 2, results in mice with impaired sociability, impaired cerebellar connectivity and motor learning, and increased propensity to engage in cognitive inflexibility (Peter et al., 2016). Finally, mutant “Lurcher” mice that experience post-natal degeneration of more than 90% of the cerebellar PCs

are characterized by distinct and severe ataxia, resulting in a recognizably disrupted gait (Caddy & Biscoe, 1979).

The aforementioned studies suggest phenotypes specific to ASD, such as impaired sociability, presence of stereotyped, repetitive behaviors, and deficits in motor control and motor learning capabilities, may be the result of altered circuitry of the cerebellar cortex. Loss of cerebellar PCs has been proposed to be the most consistent neuropathological finding in human populations of those with ASD (Fatemi et al., 2012; Hoxha et al., 2017). PC loss, ranging from a 35-95% reduction in this cellular population, has been reported within many post-mortem studies of those with ASD, those remaining appearing smaller than those in typically developing individuals (Fatemi et al., 2002; Mosconi et al., 2015).

The role of intact or disrupted cerebellar circuitry remains a component of ASD directed research. PC, parallel fiber and PC, climbing fiber synaptic connections are associated with the motor learning process, however, exact mechanisms remain to be elucidated. Current understanding is that erroneous signals are interpreted from external stimuli, then relayed through olivary climbing fibers in the cerebellar cortex (Mosconi et al., 2015). Excitation increases glutamate release from olivary climbing fibers, activating and assisting with modulation of PC signaling to deep cerebellar nuclei (Mosconi et al., 2015). Activity based alterations in synaptic signaling, synaptic plasticity, can result in stabilization of synapses as well as synaptogenesis, the formation of new synaptic connections, when neuronal activation is in synchrony (Black, Isaacs, Anderson, Alcantara, & Greenough, 1990; Hebb, 1950). Therefore, implicit, procedural motor learning with synchronous neuronal activation may induce connectivity alterations within the cerebellum, specifically between PCs and olivary climbing fibers.

Alterations in cerebellar connectivity have been noted within humans with ASD experiencing deficit in motor learning. Those with ASD display decreased cerebellar activation, decreased glutamatergic stimulation, when compared to typically developing patients (Mostofsky et al., 2009). Those with ASD may display disordered synaptic plasticity, providing a potential mechanism for motor learning deficit (Jeste, 2011). Investigation involving the 15q11-13 copy number variant mouse model for ASD detected dysregulation at PC, parallel fiber synapses (Piochon et al., 2014). With PC, climbing synaptic networks playing a critical role in motor learning aberrant cerebellar connectivity is a potential driving force for phenotypic alterations associated with or innate to ASD (Nguyen-Vu et al., 2013).

Serotonin in Cerebellar Development, Possible Relevance to Autism Spectrum Disorder

Serotonin is an indolamine developmental factor and neurotransmitter that contributes to neurological development and function of various regions throughout the brain, such as the cerebellum. However, the exact physiological and functional significance of serotonin has yet to be entirely elucidated. It is one of the earliest functioning developmental factors within the mammalian brain, likely due to its abundance throughout early neuronal development (Whitaker-Azmitia, 2001). Serotonin is a growth factor throughout embryogenesis, contributing to the development of the central nervous system with dendritic elaboration, synaptogenesis, neurogenesis, and autoregulation of the serotonergic system (Hough & Segal, 2016; Whitaker-Azmitia, 2001).

The cerebellum receives particularly rich innervation by serotonergic fibers. These projections comprise the third largest afferent fiber population within the region. Additionally, most cerebellar cell populations transiently express a multitude of serotonin

receptor subtypes at various developmental windows (Hoxha, Tempia, Lippiello, & Miniaci, 2016). Serotonergic control of cerebellar development occurs within the first three weeks of life in rodents and within the first two years in humans, providing distinct periods of developmental vulnerability (Oostland & van Hooft, 2013). Cerebellar maturation progresses through three main stages: dendritic growth and synapse formation, dendritic growth suppression and stimulation of synaptic plasticity, and synapse stabilization.

Throughout rodent postnatal week one, 5-HT₁ receptors are activated on granule cells and PCs within the cerebellum, stimulating PC dendritic arborization and synapse formation. The next week, 5-HT₁ activation begins to recede and 5-HT₃ activation on granular cells reaches maximum levels (Oostland & van Hooft, 2013). Continuous activation of the 5-HT₃ receptor, this receptor expression associated with morphological maturation of PCs, triggers secretion of reelin protein, suppressing dendritic growth (Oostland, Sellmeijer, & van Hooft, 2011). Synaptic plasticity at PC, parallel fiber and PC, climbing fiber synapses is also modulated by 5-HT₃ receptor activation. This assists in pruning of excess climbing fibers, resulting in one climbing fiber per PC once the cerebellum is fully developed (Oostland & van Hooft, 2013). Climbing fiber pruning is vital for appropriate connectivity required for motor learning and motor coordination. Throughout week two, 5-HT₂ receptor activation on granule and PCs has also begun, assisting with dendritic growth suppression. By postnatal week three, 5-HT₁ and 5-HT₃ activation has significantly decreased with 5-HT₂ receptor activity being the primary source of serotonergic activation. This results in a continued suppression of PC dendritic growth and increased synaptic stability (Oostland & van Hooft, 2013).

Developmental alterations in serotonin concentration cause morphologically variant cell populations as well as altered behavioral phenotypes within affected populations, suggesting that

specific serotonin levels are critical for typical cellular and behavioral development (Adamsen et al., 2014; Alzghoul et al., 2012; Kane et al., 2012; Mostofsky et al., 2000; Sprowles et al., 2016). Sprague Dawley rats exposed to citalopram, a nonspecific selective serotonin reuptake inhibitor (SSRI), throughout embryonic and postnatal development were reported to display behavioral phenotypes similar to ASD throughout adulthood (Sprowles et al., 2016). There has also been increased risk of conceiving a child with ASD associated with taking SSRIs or valproic acid (VPA), both pharmacologically increasing circulating serotonin concentrations (Kinast, Peeters, Kolk, Schubert, & Homberg, 2013). Additionally, mice genetically depleted of monoamine oxidase-A enzyme (MAO-A), the enzyme responsible for serotonin degradation, display increased concentrations of brain serotonin throughout cerebellar development. Such mice have abnormally developed cerebellum, presenting with a loss of PCs, reduced PC dendritic arborization, a generalized reduction of brain volume, and altered gait and motor coordination (Alzghoul et al., 2012). These suggest alterations of serotonin concentration throughout embryological and postnatal maturation may play a role in the development of ASD.

It has been proposed that increased circulating, platelet bound serotonin within a pregnant mother, perhaps from pharmacological agents, like SSRIs/VPA, or illicit drug use, could create an embryological environment with superfluous serotonin concentrations. Early in development, prior to the formation of the blood-brain barrier, excess serotonin can freely enter the central nervous system, penetrating and affecting development of various neuronal structures (Hough & Segal, 2016; Whitaker-Azmitia, 2005). As discussed previously, the cerebellum heavily relies on serotonergic influence for appropriate maturation. Therefore, this structure or the circuitry contained within it may be altered, creating phenotypic alterations characteristic of ASD. Additionally, consistent exposure to high serotonin concentrations during developmentally

vulnerable periods, the induction of developmental hyperserotonemia (DHS), could result in negative feedback of the serotonergic system, ultimately resulting in decreased concentrations of neuronal serotonin throughout adulthood, with chronically high levels of circulating serotonin.

Animals induced with DHS appear to display some of the core features of ASD, such as altered auditory and tactile sensory processing and the presence of stereotyped, repetitive behaviors (Kahne et al., 2002). Mice with genetically altered serotonin transporter proteins, resulting in increased circulating serotonin, also display altered behavior such as decreased exploration and compromised motor coordination reflected by decreased agility (Ellegood et al., 2018). Additionally, mice genetically depleted of brain serotonin, the effect of DHS induction, show reduced interest in sociability, reduced social communication reflected by decreased investigation of urine from a mouse in estrous, and increased repetitive behaviors such as marble burying, nest shredding, and digging (Kane et al., 2012). This suggests that cellular and molecular alterations may translate into altered phenotypic expression observed in those with ASD. Animals induced with hyperserotonemia of the brain, the opposite effect observed in those induced with systemic DHS, appear to display motor impairment, compromised motor learning capabilities, and a reduction in sociability (Tanaka et al., 2018). It is possible that alterations of serotonergic levels within the brain, either increased or decreased compared to controls, could be sufficient to produce the associated behavioral abnormalities. Such studies propose that induction of serotonergic system alterations may provide valid animal models for complex human disorders like ASD.

Increased circulating serotonin is the most consistent neurochemical finding within populations of human individuals with ASD. Approximately one third of affected individuals experience a 40-70% increase of platelet bound serotonin concentration (Hough & Segal, 2016;

Whitaker-Azmitia, 2001). These findings collectively suggest a common mechanism for the heterogeneous population of those with ASD, altered serotonergic systems. However, additional research must be performed within animal and human populations prior to establishing definitive conclusions.

Aims & Hypotheses

This project was designed to investigate motor function, motor learning, and cerebellar circuitry within a DHS induced Sprague Dawley rat model of ASD. Three specific research aims were addressed. The first aim was the assessment of whether the induction of DHS effects Sprague Dawley rat motor coordination when compared to saline treated control animals, reflective of the typically developing rat population. This aim was assessed by implementation of balance beam motor testing, the procedure described within the following materials and methods. It was hypothesized that rat pups induced with DHS would display a deficit in motor coordination, manifested as the animals requiring greater time to traverse a balance beam.

The second aim was to assess whether DHS induction retards motor learning within affected Sprague Dawley rat populations when compared to saline treated controls. Additionally, this aim served to investigate whether implementation of an early motor training regimen is sufficient to rescue a deficit in motor coordination within affected animals. Motor learning was evaluated by statistical analysis of balance beam motor testing and prior implementation of an early balance beam motor training regimen. Motor training is anticipated to result in improvement on a motor task, reflected by decreased traverse time, with repeated exposure. Therefore, the extent of improvement between groups was used as an assessment of motor learning. Early balance beam motor training regimen was implemented for approximately half of

the animals induced with DHS and approximately half of the control animals. This resulted in four distinct animal groupings: trained saline treated, trained DHS induced, untrained saline treated, and untrained DHS induced animals. Two hypotheses were created to address the second aim. The first was that DHS induced rat pups would be observed to have less improvement in completion of a balance beam motor test across repeated trials, the extent of reduction of traverse time being less when compared to controls. The second hypothesis was that trained DHS induced animals would be observed to have equivalent or increased improvement in completion of a balance beam motor test when compared to untrained saline treated animals. Such phenomenon would be deemed a rescue of deficit in motor coordination.

The final aim of this project was to investigate whether DHS induction and/or the implementation of an early motor training regimen within Sprague Dawley rats resulted in alterations of cerebellar cortex circuitry, specifically connectivity between PCs and olivary climbing fibers. As previously discussed, compromised motor coordination and motor learning are frequently reported within populations affected by ASD. Additionally, both phenotypes are potential manifestations of altered circuitry within the cerebellar cortex, which may be modified by alterations of the serotonergic system. It has also been proposed that procedural, implicit motor learning induces alterations in cerebellar connectivity, specifically between PCs and olivary climbing fibers. Therefore, connectivity between the two aforementioned cell populations was further investigated using immunohistochemistry techniques. Anti-calbindin antibodies, specific to PCs, and anti-VGlu2 antibodies, specific to glutamate vesicular transporter protein 2 at PC, olivary climbing fiber synapses, were employed. It was hypothesized there would be a reduction of PC, olivary climbing fiber synaptic connections, illustrated by a reduction of VGlu2 positively stained particles, within DHS induced animals. Such reduction could explain a

change in motor coordination, due to reduced adaptation to erroneous signals while completing a motor task. Additionally, it was hypothesized there would be reduced positively stained VGlut2 particle area, reflective of reduced synaptic glutamate release and/or glutamate receptor expression, within DHS induced animals. Finally, with the implementation of an early motor training regimen, it was hypothesized trained animals would displayed increased connectivity, reflected by increased positively stained VGlut2 particle counts, as well as increased synaptic glutamate release and/or receptor expression, illustrated by increased positively stained VGlut2 particle area.

MATERIALS & METHODS

Experimental Animals

All live vertebrate animal use was performed with Institutional Animal Care and Use Committee (IACUC) approval, IACUC ID: 16-034.0 approved 10/17/2016. Four timed-pregnant Sprague-Dawley rats, purchased from Charles River Laboratories, and their litter pups were utilized as the cohort for this study. Charles River Laboratories confirmed pregnancy through visualization of a vaginal plug. The first day of vaginal plug observation was considered gestational day zero (GD 0) and was used as the start date of the experimental timeline. All animals were housed within the Missouri State University, Temple Hall vivarium. Pregnant dams and progeny were housed separately in identical polycarbonate cages with water and standard rodent chow, freely available. Cages were stored within temperature (21-24 °C) controlled rooms with a 12 hour light/dark cycle. Dams were housed individually, then with their successive litters, until postnatal day (PND) 21. Pups were then divided into new groupings, ensuring for an equal distribution of males and females in each group.

Two of the four pregnant dams, 1.1 and 1.3, were randomly selected to experimental group (Figure 4) where they, and their litters, received daily subcutaneous injections of 5-methoxytryptamine (5-MT) solution. The remaining two pregnant dams, 1.2 and 1.4, and their litters, were assigned to receive daily subcutaneous injections of saline solution. Numerical tattoos at the base of their tail were applied on PND 1 for identification of all pups used in this study. Individual daily weights and administered injection dosage were recorded. Dams were euthanized on PND 21 using carbon dioxide, followed by cervical dislocation, in a room separate

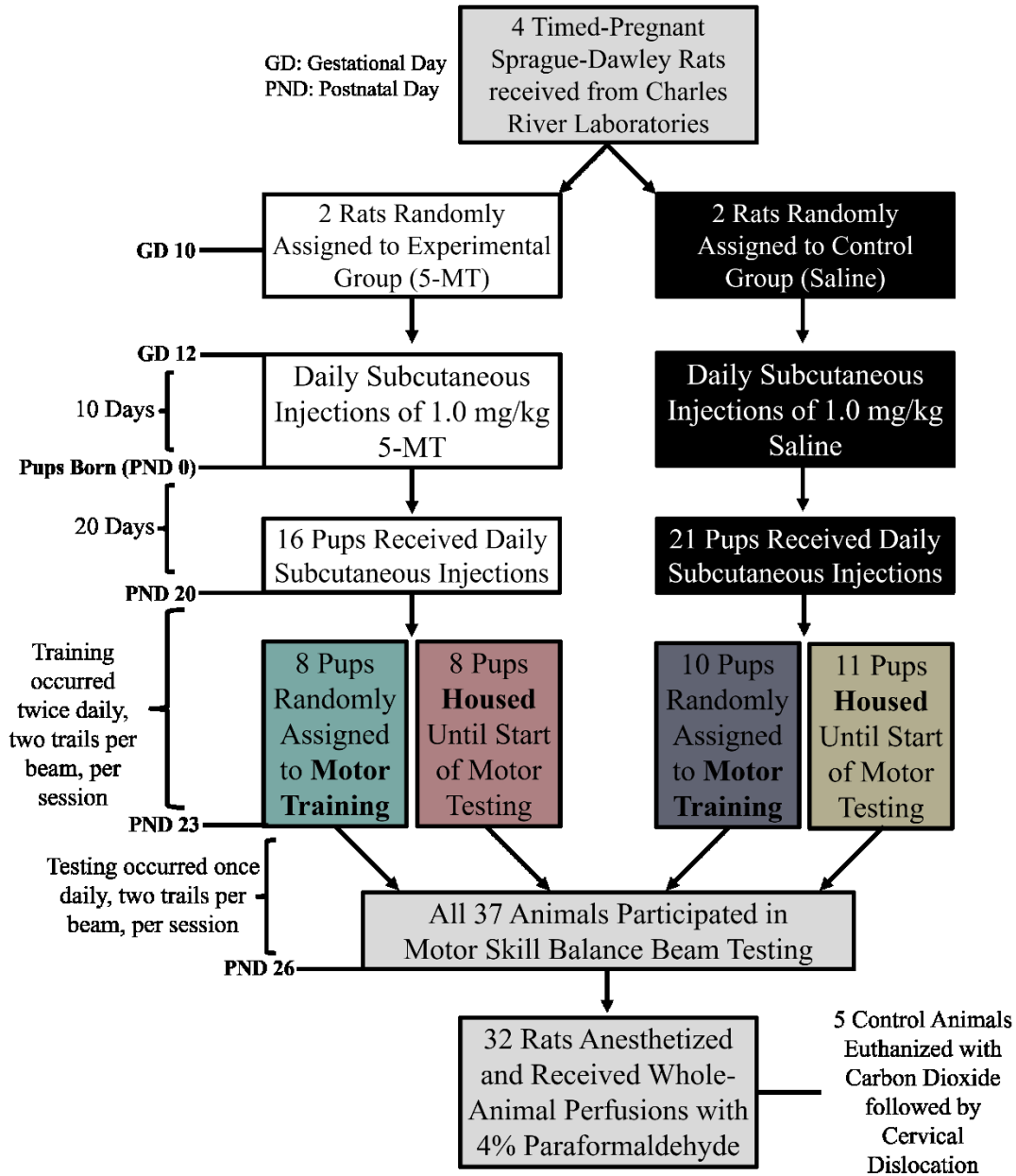


Figure 4: Research Timeline Flowchart, Including Days of Motor Training and Motor Testing. Sprague Dawley rats were subdivided into four groups, each designated by a different color. Animal groups differed in both treatment and training. Varying treatment included the induction of DHS by subcutaneous injections of 5-methoxytryptamine (5-MT) or the administration of normalized saline for controls representative of the typically developing population. In regards to training, there was either implementation of balance beam motor training or lack thereof. All groups participated in motor skill balance beam training on postnatal day 23 through postnatal day 26. All animals were then euthanized by whole-animal perfusion of 4% paraformaldehyde or carbon dioxide followed by cervical dislocation.

from remaining pups to minimize stress. Pups received additional tattoos on PND 22 due to fading of initial tattoos.

Injection Preparation & Administration

The injection administration and drug selection procedure described within this project was modified from Whitaker-Azmitia (2001), in which an animal developmental hyperserotonemic model of ASD was created. Additionally, dosage consistently remained at 1.0 mg/kg of animal body weight, in order to most closely replicate the 50% increase in circulating serotonin most commonly observed within individuals with ASD, discussed in Whitaker-Azmitia (2005).

Nonspecific serotonin agonist 5-MT solution and 85% normalized sodium chloride solution were prepared and aliquoted into sterilized 1.5 microcentrifuge tubes, covered in laboratory grade aluminum foil, then stored at -20 °C in preparation of administration of daily subcutaneous injections. Solution preparation occurred within a dimly lit room, to minimize the degradation of 5-MT, a known light sensitive compound. The 5-MT solution was prepared at a concentration of 1 mg/mL with granular 5-MT (Sigma Aldrich, Cat. No. 286583) in 85% normalized saline (Ricca Chemical Company, Cat No. 7200-32), vigorously vortexing until completely dissolved. Saline solution consisted solely of the purchased 85% normalized saline solution (Ricca Chemical Company, Cat No. 7200-32).

Randomly assigned experimental and control dams received daily subcutaneous injections between 10:00 am -12:00 pm CST of either 5-MT or normalized saline solution, respectively. Injections began on GD 12, in accordance to the developmental timeframe associated with the first emergence of serotonergic neurons. This continued until delivery of

subsequent rat pups, of which received injections coordinating with that the originating dam received, through weaning on PND 20. All injections occurred within the Missouri State University vivarium within Temple Hall in the 10:00 am – 12:00 pm CST time frame. Animals were injected while alert, without anesthesia, with solution warmed to approximately 40 °C. Ample care, in which the animals were gently, but firmly held on a steady surface as dosage was administered directly into the scruff of the neck, served to reduce stress and discomfort. Disposable, 1.5 mL plastic syringes with 25G needles were used for animals over 15 mg, including dams and older pups. 50 µL glass Hamilton syringes with 30G needles were used for animals below 15 mg of body weight.

Behavioral Testing, Assessment of Motor Function

Pups were divided into four groups upon being weaned from dams on PND 21 (Figure 4). Two of the four groups contained pups from normalized saline injected dams, and received subsequent normalized saline injections starting on PND 1; such were referred to as saline treated animals. The saline treated rat pups were then further divided into those that underwent motor skill training prior to motor skill testing, trained saline treated animals, and those that did not, untrained saline treated animals. The other two of the four animal groupings contained pups from 5-MT injected dams, and received subsequent 5-MT injections starting on PND 1; such animals were referred to as rats induced with DHS. Half of the DHS induced rats underwent motor skill training prior to motor skill testing, trained DHS induced pups, while the other half remained housed within their cages during this time, untrained DHS induced rats. Care was taken to ensure equivalent sex distribution between groups.

The beam walking protocol implemented throughout the motor skill training and motor skill testing within this study was modified from the methods described by Carter, Morton, & Dunnett. (2001). Balance beam training and testing has been routinely used for the assessment of motor coordination and balance within rodents (Carter et al., 2001). Additionally, the balance beam motor task has been reported to be more sensitive to slight alterations in motor coordination when compared to other motor tests, such as rotarod testing, when assessing drug induced impairment (Stanley et al., 2005). Sprague Dawley rat motor training began by preparing the balance beam apparatus (Figure 5), which consisted of a series of elevated, narrow beams suspended 50 cm from the ground with an enclosed escape platform 10 cm from the end of one side of the beam, a middle traverse distance of 80 cm, and a bright desk lamp 10 cm from the other end of the beam. The escape platform contained a box filled with bedding from the animals' cages for familiarity and there was a cushioned area directly below the elevated beams in the event of an animal falling. Four 100 cm long beams were used; two rectangular beams with 19 mm and 9.5 mm diameters and two cylindrical beams with 28 mm and 13 mm diameters. Balance beams of varying shape and diameter were implemented throughout both balance beam motor training and balance beam motor testing to increase the sensitivity of this task, allowing more accurate detection of differences in groups' performance.

Motor skill testing was performed on all groups from postnatal day 24 through postnatal day 26. This testing consisted of using the same balance beam apparatus, however, there was only one morning motor testing session per day. Animals were still timed traversing the 80 cm beams of varying diameter, obtaining two consecutive trials per beam and recording any failure to complete the trials, up to ten failures per testing session (Figure 5).

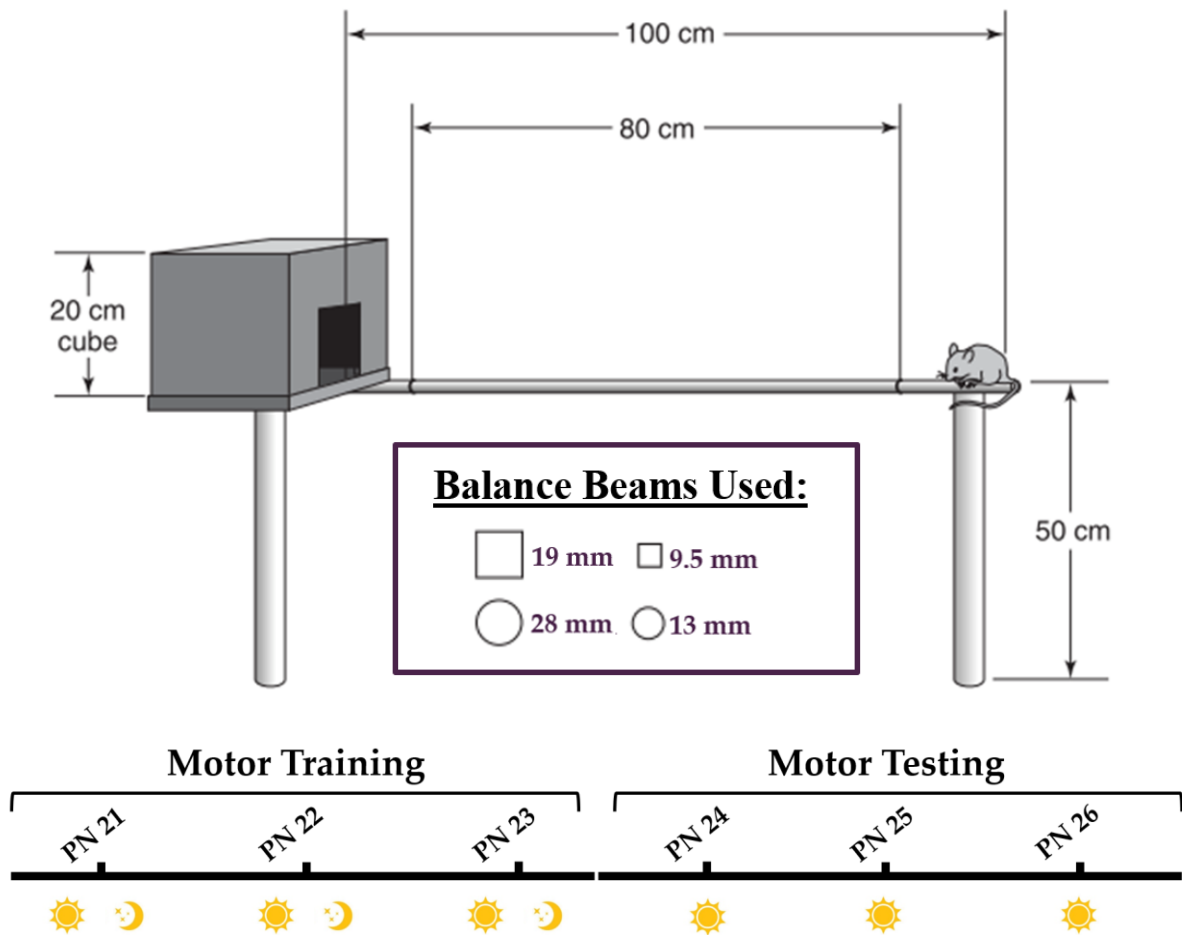


Figure 5: Balance Beam Motor Training and Testing Schematic and Timeline. Above is an illustration of the balance beam motor training and balance beam motor testing apparatus implemented within this project as reported in the 2001 Carter publication, copyright © 2001, John Wiley and Sons. The aversive, bright desk lamp positioned 10 cm from the beam, opposite to the side where the escape platform is location, is not shown. Additionally, the cushioned area directly below the balance beam motor training/testing set-up is not shown. Below the testing apparatus is a timeline representative of the motor training and motor testing timeline. Suns serve to represent morning training/testing sessions and moons represent evening training sessions.

Anesthesia, Perfusion/Fixation, & Tissue Collection

Pups were euthanized through whole-animal perfusion of a 4% paraformaldehyde solution upon completion of motor skill testing on PND 27 and PND 28. The protocol described throughout this study was modified from the methods described by Gage, et al. (2012). First, pups were placed within an induction chamber, then deeply anesthetized with 2-4% isoflurane gas at 1-2 L/min. Once the animal appeared unresponsive, it was removed from the induction chamber, placed within a metal tray, and administered a continuous supply of 2-4% isoflurane at 1-2 L/min through a rodent facemask. Appropriate anesthetization depth was confirmed through absence of hind paw withdrawal reflex to a painful toe pinch.

A medial incision of the anterior abdomen was made, moving superiorly towards the rib cage, cutting through the integument and anterior abdominal wall. Once directly inferior to the rib cage, the incision was extended laterally, the liver carefully separated from the diaphragm. Using curved blunt scissors, a traverse cut was made along the diagram. The traverse incision was continued along the entire lateral and contralateral length of the rib cage, up to the collarbone. The tip of the sternum was clamped with the tip of a hemostat, moving the hemostat over the animals head for full view of the thoracic cavity.

An incision was made into the inferior angle of the left ventricle, then a blunt tipped perfusion catheter was threaded through the left ventricle superiorly towards the ascending aorta. An additional cut was made in the right atrium to allow the release of perfusate. Slow, continuous application of pressure to a 20 mL syringe, of which the perfusion catheter was attached, allowed for approximately 40-60 mL of ice cold 25 mM phosphate buffered saline (PBS), followed by 40-60 mL of ice cold 4% paraformaldehyde to be transcardially perfused. Perfusion of ice cold 4% fixative continued until the animal achieved nuchal rigidity.

Exsanguination was monitored through continuous supervision of perfusate return through the right atrium; additionally, the liver was observed to change from deep red to pale yellow in color.

The posterior portion of the brain was then harvested from the animal, taking care to fully preserve cerebellar structures. First, traverse cuts with a razor blade were used to peel away muscle at the base of the skull. Cervical vertebrae were severed, then scissors were used to make horizontal incisions extending through the skull's foramen magnum. Rongeurs were used to carefully remove calvarium, exposing the posterior brain, including the cerebellum and brainstem. A small spatula severed the olfactory bulb and other cranial nerves from the ventral surface and the intact brain tissue was removed. The tissue was post-fixed within conical vials containing ice cold 4% paraformaldehyde and stored at 4 °C for a minimum of 12 hours.

Vibratome Sectioning

After a minimum of 12 hours of fixation within 4% paraformaldehyde solution, cerebellums were cut in the midsagittal plane using standard single edge industrial razor blades. One of the resulting two sections was returned to 4% paraformaldehyde while the other was embedded within 4% low melting point agarose created using 25 mM PBS. Molten agarose was poured into 15 x 15 x 5 mm disposable base molds (Fisherbrand Cat No. 22-363-553) and the midsagittal section was added so that the lateral portion was placed on the bottom of the mold. The molten agarose was allowed to cool at room temperature and was removed from the mold once firmly solidified. The agarose block was trimmed using standard single edge industrial blades, creating a 5 x 5 x 5 mm block that was then fixed to a metal platform with professional liquid super glue. Upon the adhesive drying, the metal platform was placed directly into the Vibratome Series 3000 specimen mount position. The specimen bath was filled with 25 mM

PBS, so that the trimmed agarose block and blade holder were covered with a thin layer of liquid. Uncoated, disposable vibratome injector blades (Polysciences Inc. Cat No. 22370) were loaded into the blade spring clamp set at a 15° angle. The vibratome speed was set to 2, amplitude set to 8.5, and the section thickness was set to 60 µm. Sagittal sections were taken from the medial surface of the midsagittal half and placed into 24-well cell culture plates (CellStar Cat No. 622 160) containing approximately 1 mL of 25 mM PBS. Section-containing cell culture plates were then stored at 4 °C until undergoing immunohistochemistry staining for visualization of PCs and climbing fiber connectivity.

Immunohistochemistry & Tissue Mounting

The following immunohistochemistry (IHC) procedure utilized within this study originated from the methods previously described by Pierce, et al. (2011). The entire protocol was performed with sagittal cerebellar sections free-floating in 24-well cell culture plates (CellStar Cat No. 622 160). First, sections were washed with 1000 µL 0.15 M glycine (BioRad Cat No. 161-0718) in 25 mM PBS and incubated within the wash solution for 10 minutes at room temperature. Sections were then incubated in 300 µL of 25 mM PBS with 0.05% Tween-20 (Fisher BioReagents Item No. BP337-100), also known as “PNT buffer”, and 1% Triton-X-100 (Millipore Sigma Cat No. 1122980101) for one hour at room temperature. Samples were washed with 1000 µL of PNT buffer for five minutes, three consecutive times. Blocking was then performed with 300 µL of 25 mM PBS, 2% donkey serum (Jackson ImmunoResearch Laboratories Cat No. 017-000-121), 1% bovine serum albumin (Fisher BioReagents Item No. EW-88057-64), 0.1% Triton-X 100, and 0.05% Tween-20. Samples remained in the blocking solution for one hour at room temperature. Anti-calbindin primary antibodies specific to PCs

(Sigma Aldrich Cat No. C9848) received a 1:5000 dilution and anti-VGlut2 primary antibodies specific to olivary climbing fibers (Millipore Cat No. AB2251) received a 1:1000 dilution, both within the previously described blocking solution. Tissue was incubated within 300 μ L of the primary antibody solution for two days on a rocker table at 4 °C. The sections were then washed with 1000 μ L of PNT buffer for five minutes, three consecutive times, holding in the third was for one additional hour.

Donkey anti-mouse AlexaFluor 594 conjugated secondary antibodies specific to the anti-calbindin primary antibodies (ThermoFischer Scientific Cat No. A-21203) received a 1:1000 dilution and donkey anti-guinea pig AlexaFluor 488 conjugated secondary antibodies specific to the anti-VGlut2 primary antibodies (Jackson ImmunoResearch Laboratories Cat No. 706-545-148 received a 1:200 dilution, both within the blocking solution. Sections were incubated with 300 μ L of the secondary antibody solution for 24 hours while on a rocker table at 4 °C. Next, the samples were washed with 1000 μ L of PNT buffer for five minutes, three times, holding in the third wash for 4-12 hours on a rocker table at 4 °C. The samples were washed with 1000 μ L PNT buffer for five minutes, twice more, then were ready for mounting and coverslipping.

Sagittal cerebellar sections were mounted onto positively charged StarFrost adhesive 1"x3" microscope slides. Sections were semi-aqueously mounted in FluoroGel II with DAPI (Electron Microscopy Sciences, Cat No. 17985-50) and coverslipped with #1.5 24x6 mm coverglass (Fisherbrand). Once the FluoroGel II with DAPI dried, clear nail polish was used to seal the coverslip edges. Nail polish was allowed to dry completely and care was taken to ensure all edges were fully sealed. Slides were then covered in aluminum foil and stored at 4 °C until imaged with confocal microscopy.

Confocal Imaging & Analysis

Immunohistochemistry staining of sagittal cerebellar sections was assessed using a Leica DMI8 inverted confocal microscope. Sealed, coverslipped slides were inverted and focused under a 10x objective lens, first using brightfield microscopy. The cerebellar primary fissure and inferior lobe were identified to serve as structural boundaries for lobules VI – IX, the lobules of interest for confocal imaging. All z-stack images were obtained from within posterior lobules VI – IX. The posterior lobe was selected for confocal imaging due to its association with motor coordination through the GABAergic suppression of involuntary movement. Additionally, the posterior lobe has become recently associated with language deficits, strengthening the likelihood of this region being altered within individuals with ASD (Stoodley et al., 2018).

Once the cerebellar posterior lobe was focused using the 10x objective lens, PCs were focused on using the brightfield 40x objective lens with oil immersion. Confocal microscopy was then used to view immunofluorescence of the blue channel DAPI (excitation set at 405 nm), red channel anti-calbindin (excitation set at 594 nm), and green channel anti-VGlu2 (excitation set at 488 nm) fluorescent signals. The pinhole aperture was set to one Airy unit, stack step-size was set to 2 μm along the z-axis, and the sampling aperture was set to a 512 x 512 pixel distribution. Additionally, care was taken to limit excessive fluorescence or brightfield exposure to samples, to prevent photobleaching of cerebellar sections. Approximately five z-stacks were obtained for every tissue section and stack images were collected by those blind to the experimental conditions of the animals sections originated from.

The acquired confocal z-stacks were then analyzed using Fiji software, specifically assessing and quantifying climbing fiber synaptic connectivity through VGlu2 immunohistochemistry staining (Schindelin et al., 2012). Leica files were directly imported into

ImageJ and settings were such that merged-channel stacks were converted into individual stacks for each fluorescent channel used throughout confocal imaging: channel 0 for blue, DAPI fluorescence, channel one for red, anti-calbindin fluorescence, and channel two for green, anti-VGlut2 fluorescence.

Channel two stacks were converted into black and white 8-bit stacks, then auto-thresholded using default settings with white objects on black background. Regions of interest for VGlut2 particle analysis included areas within the cerebellar molecular layer, avoiding assessment of PC bodies or the granule cell layer. Olivary climbing fibers maintain the ability to form synaptic connections with PCs within regions of Purkinje soma, however, this region was excluded due to concerns of mistakenly capturing VGlut2 particles within the granular layer across the entire stack's analysis. Particle analysis was set to assess VGlut2 particles ranging from 0.3 – 2.5 μm^2 ; this area range was determined by screening VGlut2 particle size within 22 stacks originating from three untrained, control animals.

Statistical Analysis

Statistical analysis of behavioral balance beam motor testing consisted of two-between, one-within ANOVAs with repeated measures on the last factor performed with IBM SPSS 20.0 for each individual balance beam implemented. A p value ≤ 0.05 was set for statistical significance between group analyses. Additionally, the 13 mm cylindrical beam was excluded from analysis due to excessive animal failures resulting in large amounts of missing data. The extent of missing data prevented appropriate statistical analysis and group comparisons to be made.

The two-between, one-within ANOVAs with repeated measures on the last factor allowed assessment of simple main effects of each independent variable on the dependent variable as well as a potential interaction between the two independent variables on the dependent variable. The first simple main effect tested was the effect of treatment on motor performance; animals received either daily subcutaneous injections of a non-specific serotonin agonist solution to induce DHS or injections of normalized saline solution to serve as representatives of the typically developing Sprague Dawley rat population. The second simple main effect tested was the effect of training on motor performance; animals either participated in a balance beam motor training regimen prior to the motor testing period or remained housed within their cage throughout this time. The interaction between treatment and training and the collective effect on motor performance was analyzed to assess motor learning. It is anticipated that typically developing animals will display improvement on a motor task with additional exposure or training, improvement reflected by a decreased time required to traverse the balance beam. Therefore, an interaction between treatment and training on motor performance would suggest that an improvement of motor performance is, in part, dependent on the treatment the animal received. This is to be interpreted as treatment having a potential effect on motor learning capabilities, creating cohort variance in motor performance improvement across motor testing trials.

Statistical analysis of glutamatergic connectivity between PCs and olivary climbing fibers within the cerebellar cortex consisted of two-way ANOVAs and two-sample t-tests performed with IBM SPSS 20.0. Two-way ANOVA allowed assessment of simple main effects of both independent variables, treatment, saline treatment or the induction of DHS, and the presence or absence of training on how many positive stained VGlut2 particles were detected with ImageJ

particle analysis. Additionally two-way ANOVAs allowed assessment of simple main effects of each independent variable on the total area of positive VGlut2 staining, as particle size varied. Two-sample t-tests were performed to assess the effects of training, combining data collected from saline treated animals and DHS induced animals, on total number of positively stained VGlut2 particles. Another two-sample t-test was performed to assess effects of treatment, combining trained and untrained animal data, on the total number of positively stained VGlut2 particles. A p value ≤ 0.05 was set for statistical significance between group analyses.

RESULTS

Rat Motor Performance & Motor Learning Assessment via Balance Beam Motor Training & Testing

Saline treated and DHS induced Sprague Dawley rats were randomly divided into four groups on postnatal day 21 (Figure 4). Two of the four groups were assigned to complete balance beam motor training, trained saline treated animals and trained DHS induced animals (Figure 5) (Carter et al., 2001). Upon completion of balance beam motor training, all four animal groups performed motor testing (Figure 5). Traverse times for each training session throughout motor training and for each day of testing throughout the motor testing period were recorded for each individual beam, averaged, and analyzed (Figure 6, 7, & 8). Results from the small round (13mm) balance beam were recorded, but excluded from statistical analysis, due to excessive failures across groups (Figure 9 & Table 1). Failure to perform was defined by the rodent falling from the beam, flipping 180°, or exceeding the maximum time allotment of 60 seconds. Untrained saline treated rats accounted for approximately 39% of the small (13 mm) round balance beam failures throughout motor testing, while untrained DHS induced rats accounted for approximately 56% of the failures (Table 2).

Two-between, one-within ANOVAs with repeated measures on the last factor, the beam used, were used to measure main effects and/or an interaction between the two independent variables, non-serotonin agonist or saline treatment, and motor training or lack thereof, on rat motor performance throughout motor balance beam testing for each individual balance beam, excluding the small (13 mm) round balance beam. Motor learning was assessed by analysis of the interaction between treatment and motor training's effect on traverse time. Control animals

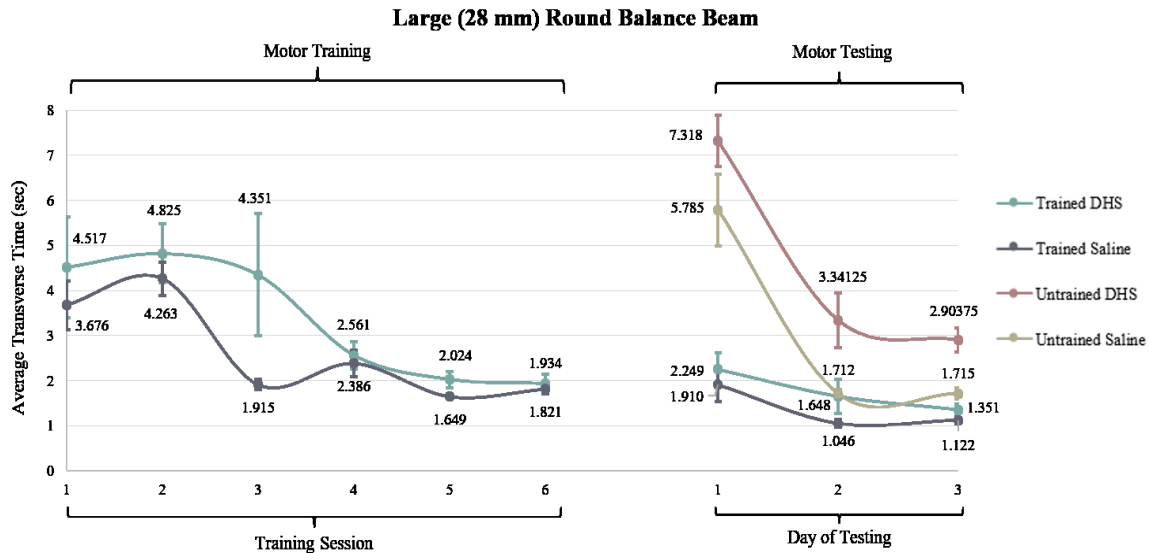


Figure 6: Mean Traverse Times Across Large (28 mm) Round Balance Beam Throughout Motor Training and Motor Testing for All Groups. Saline treated and DHS induced animals were randomly divided into four groups upon being weaned from dams on postnatal day 21, shown within the experimental schema described in Figure 4. Animals assigned to motor training, trained saline treated and trained DHS induced, performed two consecutive trials on the large, round balance beam. Large, round beam traverse times were averaged for morning and evening training sessions. The other two groups, untrained saline treated and untrained DHS induced, remained housed during this training period. Upon training fruition, all four groups performed motor testing, two trials of testing on the large, round beam per day being averaged for each animal (Figure 5). Cohort average traverse times for each training session throughout motor training and for each day of testing throughout the motor testing period are displayed above with error bars reflective of the standard error.

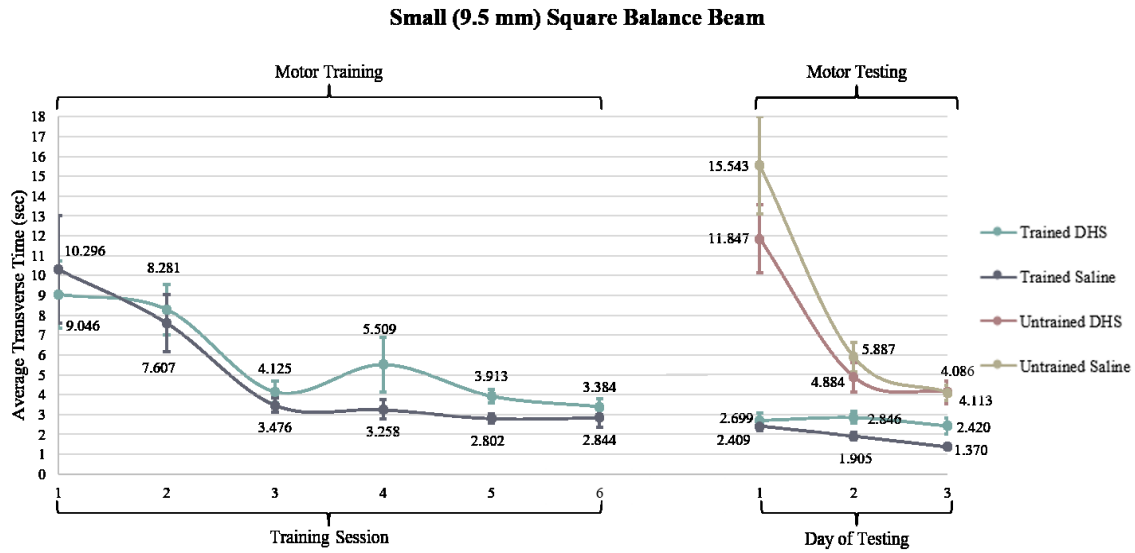


Figure 7: Mean Traverse Times Across Small (9.5 mm) Square Balance Beam Throughout Motor Training and Motor Testing for All Groups. Saline treated and DHS induced animals were randomly divided into four groups upon being weaned from dams on postnatal day 21, shown within the experimental schema described in Figure 4. Animals assigned to motor training, trained saline treated and trained DHS induced, performed two consecutive trials on the small, square balance beam. Small, square beam traverse times were averaged for morning and evening training sessions. The other two groups, untrained saline treated and untrained DHS induced, remained housed during this training period. Upon training fruition, all four groups performed motor testing, two trials of testing on the small, square beam per day being averaged for each animal (Figure 5). Cohort average traverse times for each training session throughout motor training and for each day of testing throughout the motor testing period are displayed above with error bars reflective of the standard error.

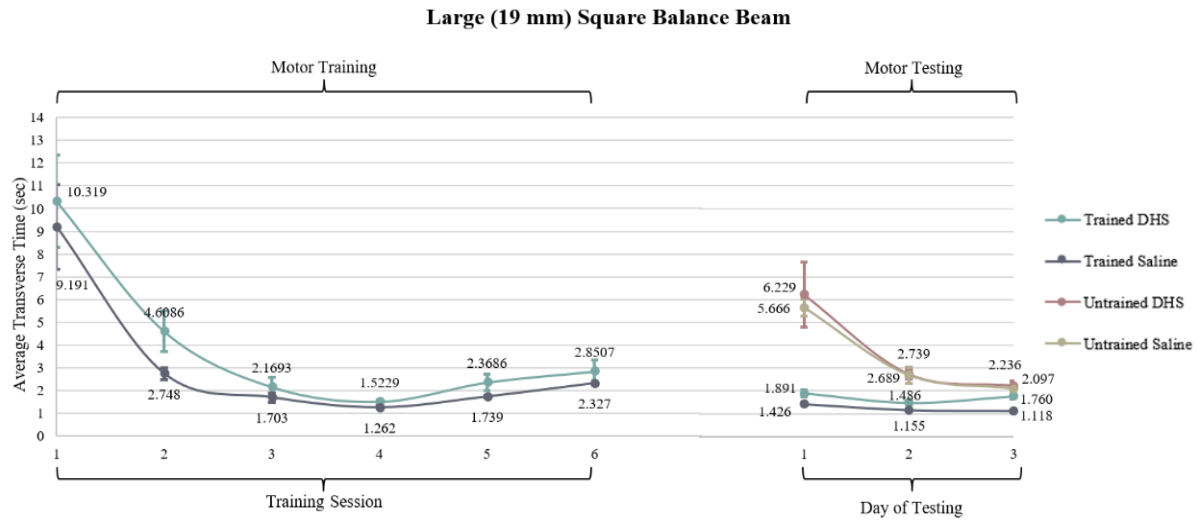


Figure 8: Mean Traverse Times Across Large (19 mm) Square Balance Beam Throughout Motor Training and Motor Testing for All Groups. Saline treated and DHS induced animals were randomly divided into four groups upon being weaned from dams on postnatal day 21, shown within the experimental schema described in Figure 4. Animals assigned to motor training, trained saline treated and trained DHS induced, performed two consecutive trials on the large, square balance beam. Large, square beam traverse times were averaged for morning and evening training sessions. The other two groups, untrained saline treated and untrained DHS induced, remained housed during this training period. Upon training fruition, all four groups performed motor testing, two trials of testing on the large, square beam per day being averaged for each animal (Figure 5). Cohort average traverse times for each training session throughout motor training and for each day of testing throughout the motor testing period displayed above with error bars reflective of the standard error.

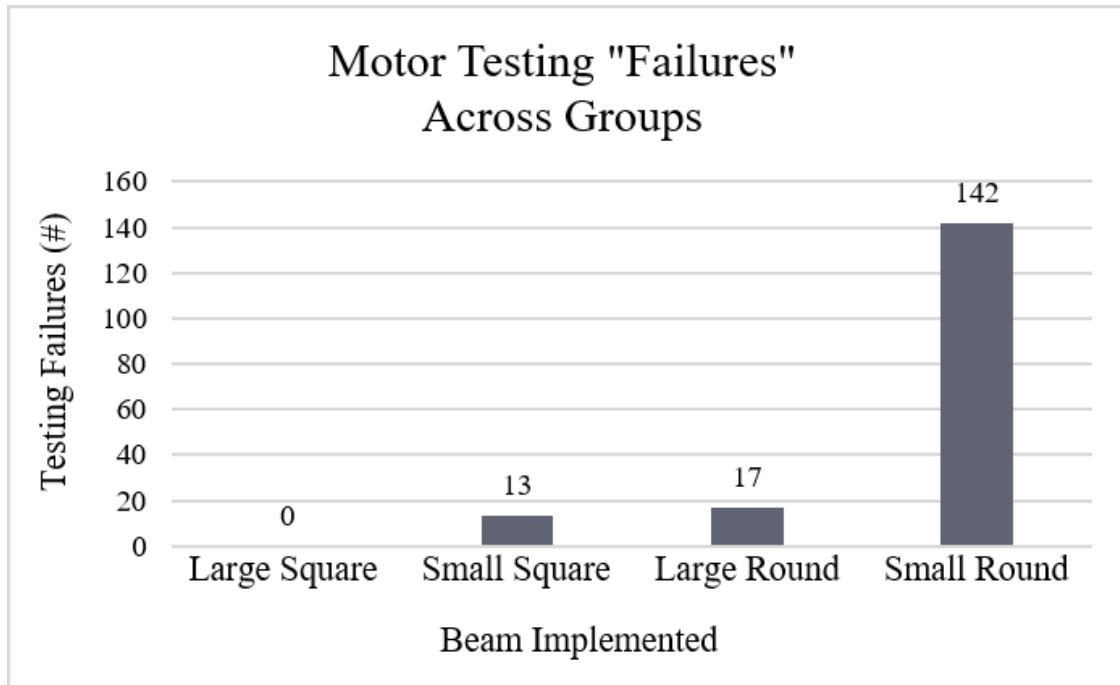


Figure 9: Trial “Failures” Acquired for Each Individual Balance Beam Throughout Balance Beam Motor Testing Across All Groups. Saline treated and DHS induced animals were randomly divided into four groups upon being weaned from dams on postnatal day 21, shown within the experimental schema described in Figure 4. All animals completed balance beam motor testing with four beams of varying shape and diameter: large (19 mm) rectangular beam, small (9.5 mm) rectangular beam, large (28 mm) cylindrical beam, and small (13 mm) cylindrical beam (Figure 5). Throughout motor testing, rats were allowed a maximum of ten failures in the attempt to achieve two successful trials per beam. A traverse time surpassing 60 seconds, the animal falling from the beam, or the animal flipping 180° on the beam were defined as failures. The total number of failures, per beam, across all groups is displayed above.

Table 1: Trial Failures for Each Balance Beam Throughout Balance Beam Motor Testing, Separated by Group.

Failures Throughout Balance Beam Motor Testing (#)				
	Large (19 mm) Square	Small (9.5 mm) Square	Large (28 mm) Round	Small (13 mm) Round
Trained Saline	0	1	0	3
Trained DHS	0	0	2	3
Untrained Saline	0	2	3	56
Untrained DHS	0	10	12	80
Total Failures/Beam	0	13	17	142

Table 2: Trial Percent Failures for Each Individual Balance Beam Throughout Balance Beam Motor Testing, Separated by Group.

Percent Failure Throughout Balance Beam Motor Testing (%)				
	Large (19 mm) Square	Small (9.5 mm) Square	Large (28 mm) Round	Small (13 mm) Round
Trained Saline	0	7.69	0	2.11
Trained DHS	0	0	11.76	2.11
Untrained Saline	0	15.38	17.65	39.4
Untrained DHS	0	76.92	70.59	56.3

should display improvement on a balance beam motor testing task across trials, with greater improvement if included in a training group prior to the testing period. Therefore, an interaction between treatment and motor training's effect on balance beam testing performance is suggestive that the extent of motor performance improvement across testing after motor training completion is, in part, dependent on the treatment the animal has received.

Upon analysis of the large (28 mm) round cylindrical beam (Figure 10), a simple main effect of treatment on motor performance (Figure 10A) ($F(1, 32) = 9.677, p = 0.004, \eta_p^2 = 0.232$) and a simple main effect of motor training on motor performance (Figure 10B & 10C) ($F(1, 32) = 57.394, p = 0.001, \eta_p^2 = 0.642$) were found to be statistically significant. However, there was no statistically significant interaction between the two independent variables, treatment and training (Figure 10D & 10E) ($F(1, 32) = 3.210, p = 0.083, \eta_p^2 = 0.091$). Analysis of the small (9.5 mm) square rectangular beam (Figure 11) revealed a statistically significant simple main effect of motor training on motor performance (Figure 11B & 11C) ($F(1, 32) = 53.914, p = 0.001, \eta_p^2 = 0.628$). There was no statistical significance found for a simple main effect of treatment on motor performance (Figure 11A) ($F(1, 32) = 0.279, p = 0.601, \eta_p^2 = 0.009$), nor an interaction between the two independent variables (Figure 11D & 11E) ($F(1, 32) = 2.406, p = 0.131, \eta_p^2 = 0.070$). Statistical analysis of the large (19 mm) square rectangular beam (Figure 12) showed similar trends to the small square (9.5 mm) balance beam with a statically significant simple main effect of motor training on motor performance (Figures 12B & 12C) ($F(1, 32) = 74.990, p = 0.001, \eta_p^2 = 0.701$) with no statistical significance regarding a simple main effect of treatment on motor performance (Figure 12A) ($F(1, 32) = 2.190, p = 0.149, \eta_p^2 = 0.064$) nor an interaction between the two independent variables (Figure 12D & 12E) ($F(1, 32) = 0.215, p = 0.649, \eta_p^2 = 0.007$).

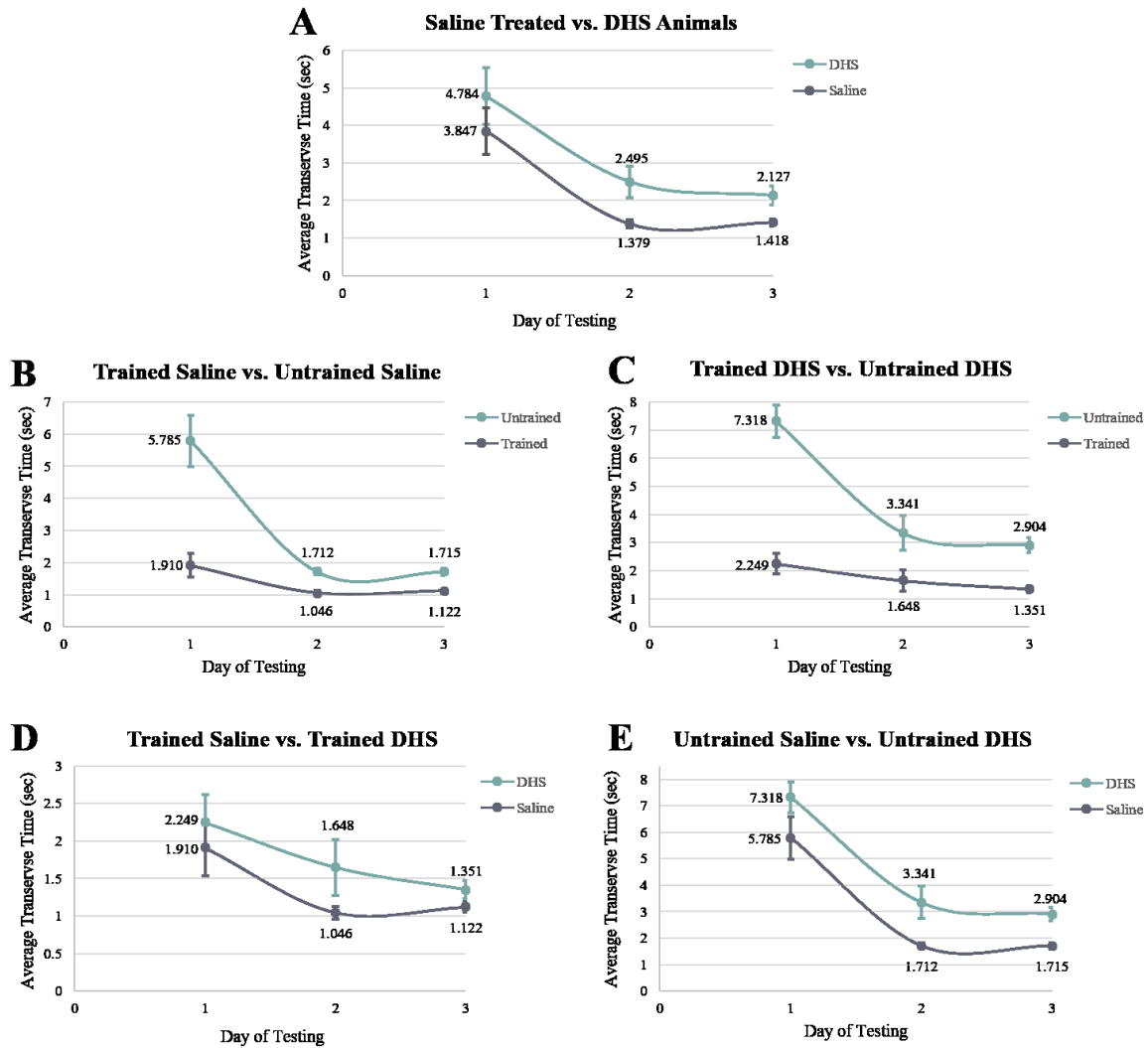


Figure 10: Large (28 mm) Round Balance Beam Analyses of Simple Main Effects and Interaction Between Treatment and Motor Training on Motor Testing Performance. Saline treated and DHS induced animals were randomly divided into four groups upon being weaned from dams on postnatal day 21, shown within the experimental schema described in Figure 4. Two-between, one-within ANOVA with repeated measures on the last factor was used to analyze the simple main effect of treatment (**A**), the simple main effect of motor training (**B** and **C**), and the interaction between both independent variables on balance beam motor testing performance (**D** and **E**). The simple main effect of treatment on motor performance throughout balance beam motor testing (**A**) was found to be statistically significant, $F(1, 32) = 9.677, p = 0.004, \eta_p^2 = 0.232$. The simple main effect of motor training on motor performance throughout balance beam motor testing (**B** and **C**) was also found to be statistically significant, $F(1, 32) = 57.394, p = 0.001, \eta_p^2 = 0.642$. However, the interaction of treatment and motor training on motor performance throughout balance beam motor testing (**D** and **E**) was not found to be statistically significant, $F(1, 32) = 3.210, p = 0.083, \eta_p^2 = 0.091$.

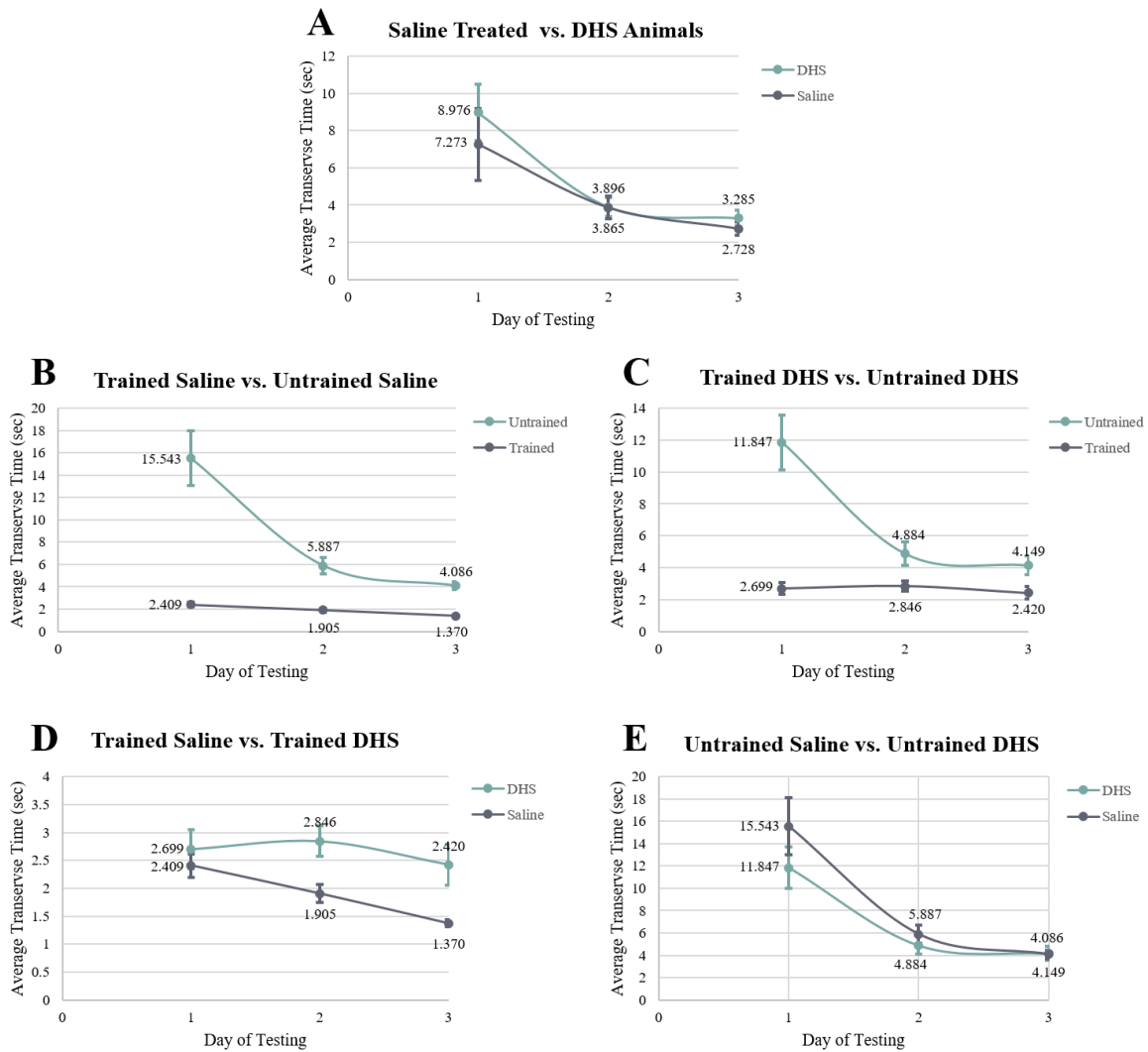


Figure 11: Small (9.5 mm) Square Balance Beam Analyses of Simple Main Effects and Interaction Between Treatment and Motor Training on Motor Testing Performance.

Saline treated and DHS induced animals were randomly divided into four groups upon being weaned from dams on postnatal day 21, shown within the experimental schema described in Figure 4. Two-between, one-within ANOVA with repeated measures on the last factor was used to analyze the simple main effect of treatment (A), the simple main effect of motor training (B and C), and the interaction between both independent variables on balance beam motor testing performance (D and E). The simple main effect of treatment on motor performance throughout balance beam motor testing (A) was not found to be statistically significant, $F(1, 32) = 0.279$, $p = 0.601$, $\eta_p^2 = 0.009$. The simple main effect of motor training on motor performance throughout balance beam motor testing (B and C), however, was found to be statistically significant, $F(1, 32) = 53.914$, $p = 0.001$, $\eta_p^2 = 0.628$. The interaction of treatment and motor training on motor performance throughout balance beam motor testing (D and E) was also not found to be statistically significant, $F(1, 32) = 2.406$, $p = 0.131$, $\eta_p^2 = 0.070$.

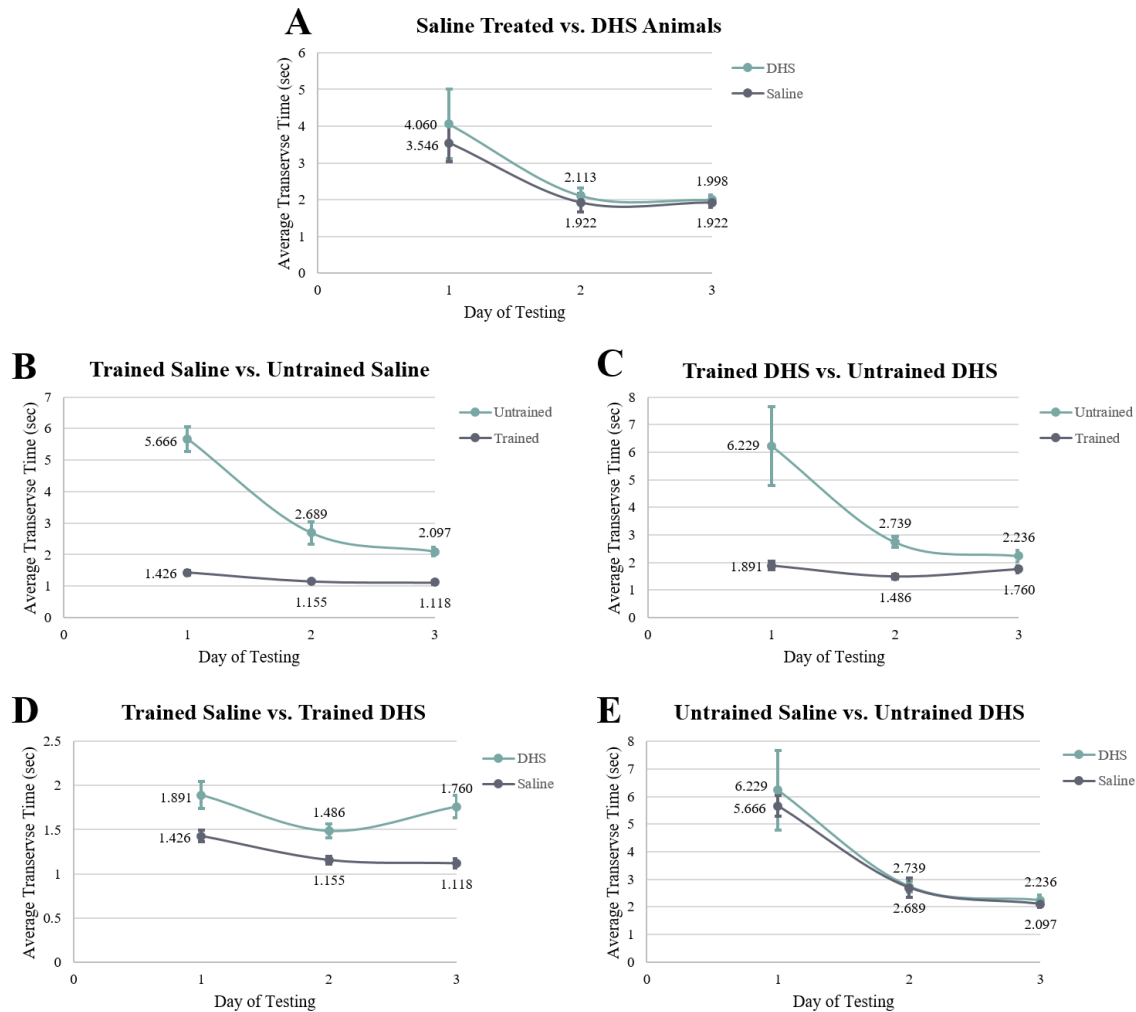


Figure 12: Large (19 mm) Square Balance Beam Analyses of Simple Main Effects and Interaction Between Treatment and Motor Training on Motor Testing Performance.

Saline treated and DHS induced animals were randomly divided into four groups upon being weaned from dams on postnatal day 21, shown within the experimental schema described in Figure 4. Two-between, one-within ANOVA with repeated measures on the last factor was used to analyze the simple main effect of treatment (A), the simple main effect of motor training (B and C), and the interaction between both independent variables on balance beam motor testing performance (D and E). The simple main effect of treatment on motor performance throughout balance beam motor testing (A) was not found to be statistically significant, $F(1, 32) = 2.190, p = 0.149, \eta_p^2 = 0.064$. The simple main effect of motor training on motor performance throughout balance beam motor testing (B and C), however, was found to be statistically significant, $F(1, 32) = 74.990, p = 0.001, \eta_p^2 = 0.701$. The interaction of treatment and motor training on motor performance throughout balance beam motor testing (D and E) was also not found to be statistically significant, $F(1, 32) = 0.215, p = 0.649, \eta_p^2 = 0.007$.

Investigation of Glutamatergic Synaptic Connectivity of Purkinje Cells & Olivary Climbing Fibers

Connectivity between PCs and olivary climbing fibers within the cerebellar cortex was investigated through application of immunohistochemistry, followed by quantification using ImageJ software, and statistical analysis. PCs were labeled with anti-calbindin antibodies and olivary climbing fiber glutamate vesicular transporter 2 proteins were labeled with anti-VGlu2 antibodies, allowing indirect assessment of glutamatergic synapses between the two neuronal cell types. The fluorescently stained sagittal cerebellar sections were imaged at an objective lens magnification of 40x with oil immersion using a Leica DMI8 confocal microscope (Figure 13). Approximately five z-stacks were obtained for each stained tissue section, with approximately nine sections being stained for each animal that was transcardially perfused with 4% paraformaldehyde. ImageJ software allowed quantification of the number of positively stained VGlu2 particles for the imaged z-stacks, each particle representative of a single synaptic connection. It also allowed quantification of the total area of positive VGlu2 staining, representative of the volume of glutamate release from an olivary climbing fiber.

Two-way ANOVA was used to statistically analyze possible simple main effects of the two independent variables, treatment and training, on the quantified number of positively stained VGlu2 particles. Treatment groups differed in that Sprague Dawley rat pups were either induced with DHS or administered subcutaneous injections of normalized saline to serve as experimental controls. Training groups differed by implementation of an early balance beam motor training regimen or lack thereof prior to balance beam motor testing.

Upon analysis of the number of positively stained VGlu2 particles, a simple main effect of treatment (Figure 14A) was found to be statistically significant when comparing trained saline

treated with trained DHS induced animals ($F(1, 32) = 27.13, p = 0.002$) as well as when comparing untrained saline treated with untrained DHS induced animals ($F(1, 32) = 26.94, p = 0.002$). The simple main effect of training (Figure 14A) was also found to be statistically significant when comparing trained saline treated with untrained saline treated animals ($F(1, 32) = 14.32, p = 0.017$) as well as trained DHS induced with untrained DHS induced animals ($F(1, 32) = 22.67, p = 0.003$). Similar two-way ANOVA statistical analysis allowed investigation of main effects of treatment and training on VGlut2 staining area (Figure 14B). It was found that the main effect of treatment was statistically significant when comparing trained saline treated with trained DHS induced animals ($F(1, 32) = 12.53, p = 0.026$) as well as when comparing untrained saline treated with untrained DHS induced animals, ($F(1, 32) = 11.23, p = 0.019$). The main effect of training on VGlut2 staining area (Figure 14B) was also found to be statistically significant when comparing trained saline treated with untrained saline treated animals ($F(1, 32) = 8.32, p = 0.032$) as well as trained DHS induced with untrained DHS induced animals ($F(1, 32) = 4.78, p = 0.035$). Quantified VGlut2 particle counts were also analyzed using two-sample *t*-tests to assess the effect of training and the effect of treatment on the number of positively stained VGlut2 particles within the cerebellar cortex. The effect of training on the number of positively stained VGlut2 particles (Figure 15A) was not found to be statistically significant when combining data obtained from saline treated and DHS induced animals ($t(32) = 1.790, p = 0.08$, SD of trained animals = 358.023, SD of untrained animals = 353.978). However, the effect of treatment on the number of positively stained VGlut2 particles (Figure 15B) was still found to be significant when combining trained and untrained animals, ($t(32) = 6.610, p < 0.001$, SD of saline treated animals = 63.89, SD of DHS induced animals = 55.045).

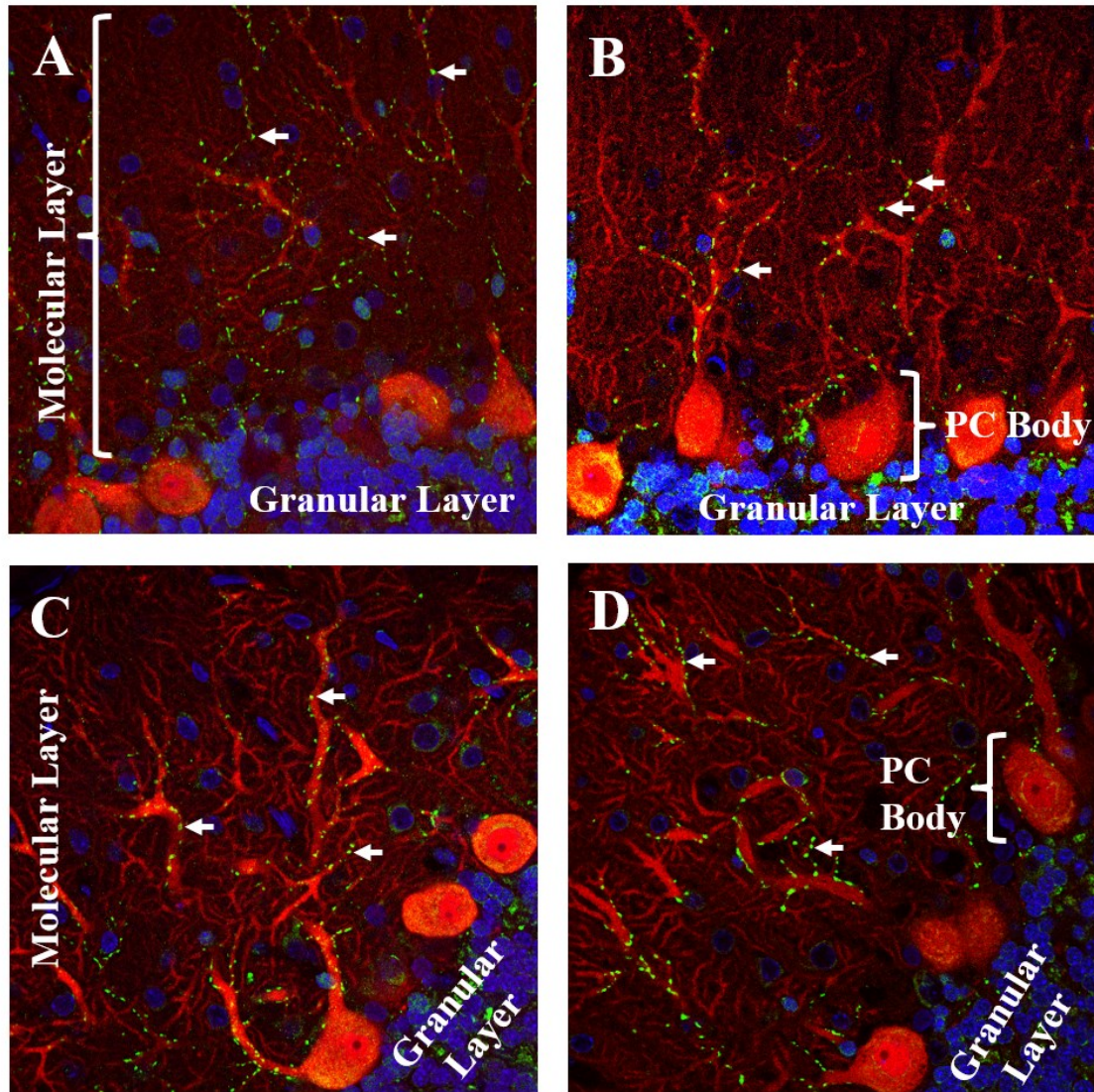


Figure 13: Representative Confocal Microscopy Images of Select Cerebellar Cortex Cell Populations within Lobules VI - IX. Cerebellar sections were imaged using a DMI8 confocal microscope at a 40x objective lens with oil immersion. Sagittal cerebellar sections were cut at 60 μm , then labeled using immunohistochemistry technique. Purkinje cells (PC) were fluorescently labeled with Alexa 594, red secondary antibodies. Olivary climbing fiber vesicular transporter protein 2 (VGlut2), representative of olivary climbing fiber synaptic connections, were labeled using Alexa 488, green. Positively stained VGlut2 particles are designed with white arrowheads. Cellular nuclei were stained using DAPI, shown as blue. There are representative images for each of the four Sprague Dawley rat subgroups, shown within Figure 3: trained saline treated (A), trained DHS induced (B), untrained saline treated (C), and untrained DHS induced (D).

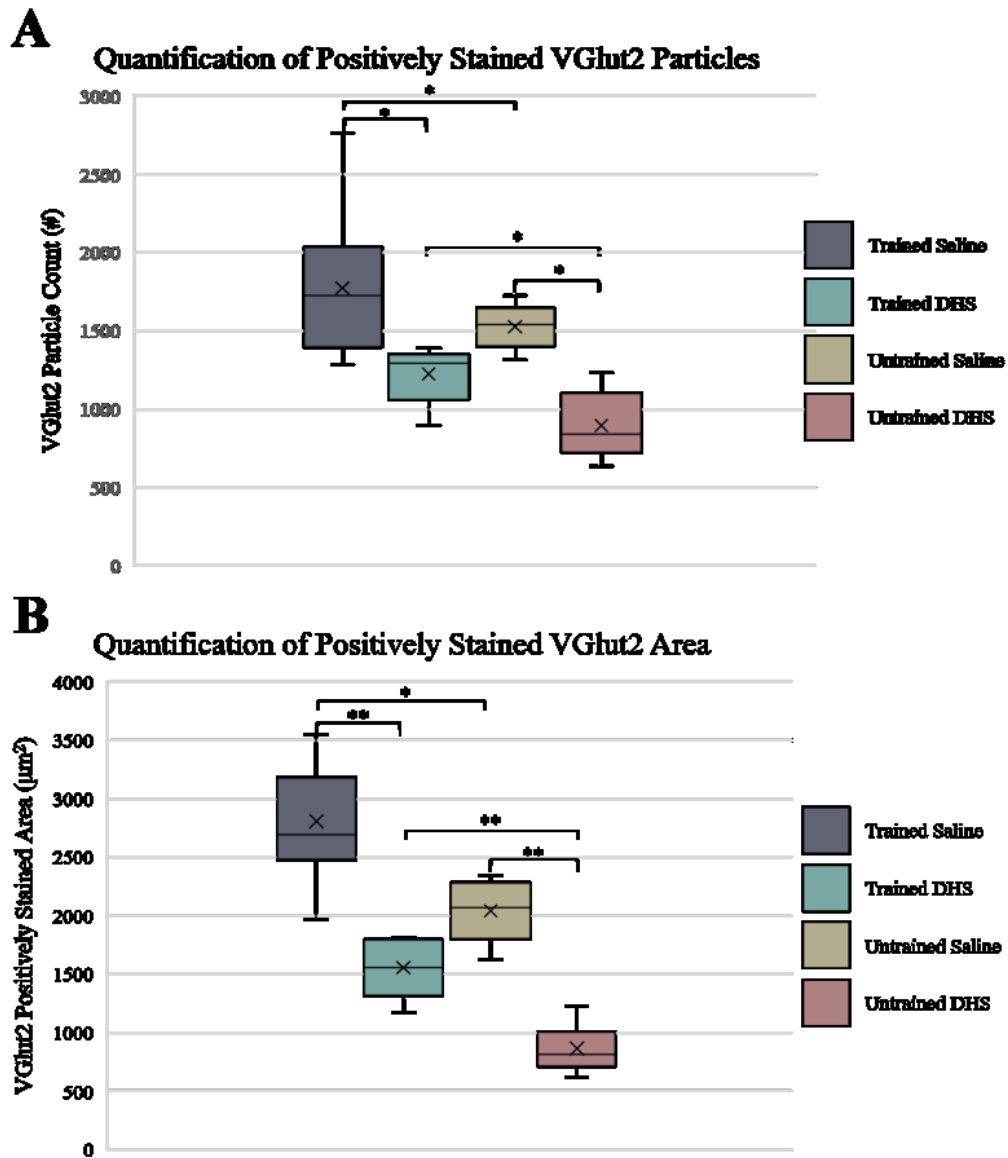


Figure 14: Quantification and Analysis of Positively Stained VGlut2 Particle Count and Area. Connectivity between PCs and olivary climbing fibers within the cerebellar cortex was indirectly assessed through the application of immunohistochemistry and ImageJ particle analysis. The number of positively stained VGlut2 particles, reflective of bound glutamate to glutamatergic receptors on Purkinje cells, were counted from z-stack images obtained from all four animal groups, shown within Figure 4, and then analyzed with two-way ANOVA. The total area of positive VGlut2 staining, reflective of volume of glutamate release, was also analyzed for all four animal groupings with two-way ANOVA. Statistical significance of $p < 0.05$ was designated with an “*”, while statistical significance of $p < 0.01$ was designated by “**”.

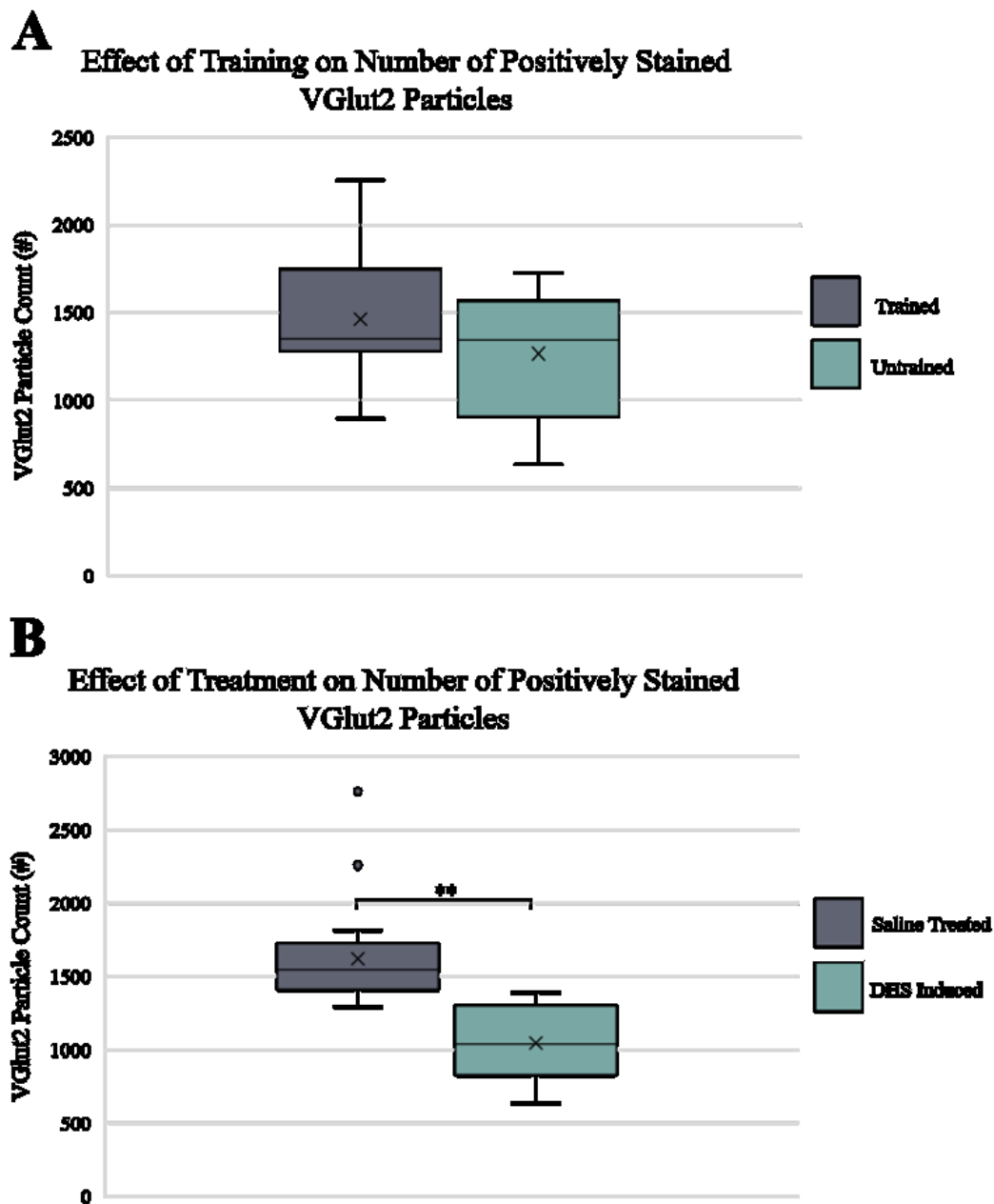


Figure 15: Assessment of Effects of Training and Treatment on Number of Positively Stained VGlut2 Particles. Connectivity between Purkinje cells and olivary climbing fibers within the cerebellar cortex was indirectly assessed through the application of immunohistochemistry and ImageJ particle analysis. The number of positively stained VGlut2 particles, reflective of bound glutamate to glutamatergic receptors on Purkinje cells, were counted from z-stack images obtained from all four animal groups, shown within Figure 4, and then analyzed by two sample t-test. Statistical significance of $p < 0.01$ was designated by “**”.

DISCUSSION

Assessment of Effects of Developmental Hyperserotonemia on Rat Motor Performance

One of the primary aims of this research is assessment of effects of DHS induction on Sprague Dawley rat motor performance. Rodent motor performance was examined through implementation of a balance beam motor task, the time required for the animal to traverse a balance beam recorded and analyzed. Four balance beams of varying shape, cylindrical or rectangular, and diameter, small or large, were used in attempt to increase motor testing sensitivity. Multiple beams allow a range of task difficulty, cylindrically shaped and/or beams of smaller diameter requiring greater motor coordination to traverse. Rounded beams also allow indirect assessment of grip, due to removal of a planar surface. Therefore, implementation of various beam shapes and diameters allows greater observation of subtle motor impairment, specifically compromised motor coordination and/or sensorimotor function (Carter et al., 2001). Affected motor coordination may be reflective of postural instability or altered gait within the DHS induced animals, as both are commonly observed within populations of humans with ASD (Bhat et al., 2011). Additionally, alterations in sensorimotor function could potentially compromise the animals' ability to integrate sensory input from external stimuli, then appropriately adjust movement in response, as is also reported in affected human populations (Paton et al., 2012). Thus, it was anticipated that the induction of DHS within a rodent population would result in decreased coordination, reflected by a greater traverse time.

Analysis of the various balance beams allowed assessment of main effects and interactions between the two independent variables, treatment and training. The simple main effect of treatment, either with the non-specific serotonin agonist 5-MT for the induction of DHS

or saline for control animals, is what was analyzed for assessment of the effects of DHS on rat motor performance. It appears that the overall trend for all analyzed beams, the large (28 mm) round cylindrical beam, small (9.5 mm) square rectangular beam, and large (19 mm) square rectangular beam is that the DHS induced animals required more time to traverse the beam than the saline treated controls (Figure 10A, 11A, & 12A). However, the large (28 mm) round balance beam was the only beam to reach statistical significance for the simple main effect of treatment (Figure 10A). The small (9.5 mm) square and large (19 mm) square beam did not reach statistical significance for this simple main effect (Figure 11A & 12A).

Such lack of statistical significance was unanticipated, for other rodent models involving disruption of the serotonergic system have been observed to display altered locomotor activity (Alzghoul et al., 2012; Noorlander et al., 2008). Additionally, balance beam motor testing has been proposed to be more sensitive to detecting reduced motor coordination when compared to other motor assays, such as rotarod behavioral testing. Therefore it was anticipated to detect even minor motor deficit if present (Stanley et al., 2005). It is possible that the lack of significance of the simple main effect of treatment for the two rectangular beams is due to small sample sizes of 8-11 Sprague Dawley pups per animal grouping, resulting in insufficient statistical power. Additionally, the combined grouping of trained and untrained animals for said analysis may have confounded results. Lastly, it is possible that more days of testing are required to detect whether motor coordination performance plateaued across all animal groups if compromised animals require more than the typical 2-3 days, on average (Carter et al., 2001). Ultimately, further research is required to identify whether motor coordination is reliably compromised within DHS induced Sprague Dawley rats, however, our data is slightly reflective of such.

Assessment of Effects of Developmental Hyperserotonemia on Rat Motor Learning

An additional primary aim of this research is assessment of effects of DHS on Sprague Dawley rat motor learning. It was predicted that induction of DHS within a Sprague Dawley rat population would retard motor learning, reflected by less improvement across balance beam motor testing trials compared to the control animals. Improvement was defined as the animal requiring less time to successfully traverse the beam with repeated task exposure. Additionally, it was hypothesized that the implementation of an early training regimen would rescue the deficit in motor coordination within the trained DHS induced animals. This motor training rescue phenomenon has been reported within a haploinsufficiency rodent model of ASD (Bachmann et al., 2019). Rescue of motor coordination was to be reflected by equivalent or greater improvement of trained DHS induced animals across motor testing trials compared to the untrained saline treated controls.

Two-between, one-within ANOVA with repeated measures on the last factor was used to assess motor learning across balance beam motor testing trials for each individual beam, excluding the small (13 mm) cylindrical beam. This allows analysis of simple main effects and interactions between the two independent variables, treatment and training. It was anticipated that the simple main effect of training on balance beam motor testing performance would be statistically significant for all beams. There is the expectation that improvement on a motor task should be observed with repetition, regardless of treatment. This hypothesis was validated in that there was a simple main effect of training found to be statistically significant for all beams analyzed, trained animals displaying reduced traverse times throughout motor testing (Figure 10B, 10C, 11B, 11C, 12B, & 12C). Such results indicate that all animals displayed propensity for motor learning.

The extent of motor learning capabilities between treatment groups was assessed by the analysis of the interaction between treatment and motor training's effect on balance beam motor testing performance. It is statistically established that all groups appear capable of motor learning (Figure 10B, 10C, 11B, 11C, 12B, & 12C). Therefore, an interaction between treatment and motor training suggests the extent of motor performance improvement across testing is, in part, due to the treatment the animal received. A possible interpretation of this could be that motor learning capabilities have been altered within some cohorts of varying treatment, creating cohort variance in motor performance improvement across the testing period.

It appears that the overall trend for all analyzed beams illustrates trained DHS induced animals required more time to traverse the beam than the trained saline treated controls, as predicted (Figure 10D, 11D, & 12D). However, with none of our analyses reaching statistical significance, one cannot definitively conclude confirmation of our hypothesis. Similarly, while trends suggest untrained DHS induced animals require additional traverse time compared to untrained controls, statistical significance was not reported (Figure 10E, 11E, & 12E). Finally, group averages suggest trained DHS induced animals performed slightly better than untrained saline treated controls on two of the three analyzed beams (Figure 6, 7, & 8). However, these trends did not reach statistical significance (Figure 10D, 10E, 11D, 11E, 12D, & 12E).

There are many possible clinical implications surrounding the use of early intervention motor training to improve compromised motor function and motor learning capacity later in life. It has been reported that individuals affected with ASD who participate in biofeedback based balance training display improved balance, reflected by increased time one can remain balanced on one foot (Travers et al., 2018). Additionally, it appears the improved balance is functionally generalized to improvement of postural based stability demonstrated while performing tasks

participants were not specifically trained on (Travers et al., 2018). Considering the established relationship between motor development and communicative development in addition to the association of motor impairment and stereotyped behaviors, there is potential that improved motor skills and motor learning may reduce core ASD symptom severity.

While these trends are observed in our investigation, the lack of statistical significance is likely the result of small sample sizes reducing the amount of statistical power for analysis. It is also possible that, again, the period of motor testing needs to be extended to better ensure that animal performance is reaching baseline, reflected by a plateauing of traverse times. While typically developing animals require an average of 2-3 days of balance beam motor testing to display plateaued traverse times, compromised animals may require further opportunity for improvement (Carter et al., 2001). Ultimately, without statistical significance one cannot reliably ascertain if a motor learning deficit was present within DHS induced animals, nor whether motor rescue is possible with establishment of an early training regimen. However, this study provides encouraging groundwork deserving of further investigation.

Assessment of Glutamatergic Synaptic Connectivity of Purkinje Cells & Olivary Climbing Fibers

The final primary aim of this research is assessment of effects of DHS and/or implementation of an early intervention motor training regimen on cerebellar cortex circuitry, specifically connectivity between PCs and olivary climbing fibers. Connectivity was assessed in two ways, one being the number of VGlut2 positive particles quantified upon analysis of fluorescently labeled cells. Each positively stained VGlut2 particle was interpreted to represent a single glutamatergic synaptic connection between a PC and olivary climbing fiber, due to the

anti-VGlut2 antibodies staining for a glutamate vesicular transport 2 protein, bound to PC glutamatergic receptors found at these synapses. It was predicted that there would be a reduction of glutamatergic synaptic connections between PCs and olivary climbing fibers with induction of DHS in Sprague Dawley rat populations. It was also hypothesized that implementation of early motor training would result in increased synaptic connections between the aforementioned neuronal cell types. Both hypotheses regarding connectivity between PCs and climbing fibers were found to be statistically significant upon analysis (Figure 14A). Cerebellar circuitry was also assessed with quantification of the total area of positive VGlut2 staining, representative of glutamate receptor abundance and/or volume of glutamatergic release. It was predicted that there would be a reduction in positively stained VGlut2 area within rats induced with DHS and that there would be an increase in positively stained area within animals that participated in early balance beam motor training. These predictions were both found to be statistically significant (Figure 14B).

The decrease of synaptic connectivity between PCs and olivary climbing fibers with animals induced with DHS suggests alterations to the serotonergic system throughout fetal and postnatal development may compromise cerebellar connectivity (Figure 15B). This is likely due to the association of serotonin with postnatal cerebellar development (Purves et al., 2001). Transient 5-HT₃ serotonin receptor expression and activation at PC, olivary climbing fiber synapses assists with the pruning of excess olivary climbing fibers (Purves et al., 2001). Therefore, indirect overstimulation of these receptors using nonspecific serotonin agonist 5-MT may overstimulate this pruning, reducing synaptic connectivity. Additionally, another animal model for ASD involving treatment with VPA, a common anti-convulsant, throughout embryonic development has been associated with increased serotonin concentration in various

brain regions, including the cerebellum (Kinast et al., 2013). Such animals display PCs with compromised dendritic arborization, altering synaptic transmission as observed in electrophysiological recordings (Wang et al., 2018). Reduced PC dendritic arbor complexity, as described, could physically limit availability to form synaptic connections. Therefore, induction of DHS may reduce PC, olivary climbing fiber synaptic connections due to excessive olivary climbing fiber pruning and/or morphological alterations to PC dendritic arbors. This is a potential cellular cause for motor dysfunction within DHS treated animals, for olivary climbing fibers assist with the integration of external stimuli, allowing modulation of motor movement (Paton et al., 2012).

Such conclusions are also supported by analysis of positively stained VGlut2 particle area, reflective of volume of glutamate release and/or receptor abundance at PC, olivary climbing fiber synapses. Animals induced with DHS display reduced positively stained VGlut2 particle area (Figure 14B & 15B). This may be due to reduced VGlut2 particle numbers decreasing overall positive staining area. However, such results also suggest cerebellar dysfunction from reduced cerebellar activation, displayed by reduced glutamate release (Mostofsky et al., 2009). This supports the notion of decreased motor learning capabilities within a DHS induced animal model for ASD. Such findings assist with validation of the DHS animal model for ASD as it reflects, on a cellular level, the reduction of motor learning and cerebellar activation reported in affected human populations (Mostofsky et al., 2009).

Conversely, increased connectivity and glutamatergic receptors and/or glutamate release with implementation of an early motor training regimen is likely reflective of increased motor learning. Motor learning was behaviorally established within all animal groups, in that all animals improved on a balance beam motor task with repeated exposure (Figure 10B, 10C, 11B,

11C, 12B, & 12C). This behavioral data is supported by our cellular findings (Figure 14A), for motor learning is associated with cerebellar synaptogenesis, the formation of additional synapses, with PCs (Black et al., 1990). Increased PC, olivary climbing fiber synapses could provide explanation for the increased positively stained VGlut2 area (Figure 14B), for more particles could result in greater overall staining area. However, it is also possible that motor learning increased cerebellar activation, displayed by increased glutamatergic stimulation of PCs (Mostofsky et al., 2009). Therefore, it appears that all animals are capable of motor learning, reflected by cerebellar circuitry alterations. Additionally, implementation of early motor training regimens increases PC, climbing fiber connectivity and glutamate release and/or receptor expression.

CONCLUSIONS & FUTURE DIRECTIONS

The heterogeneity of ASD has provided challenges in identification of reliable behavioral, structural, and/or neuronal connectivity commonalities within populations affected with ASD. The majority of individuals with ASD present varying severity regarding not only core features of the disorder, but also frequently observed comorbidities of additional diagnoses and symptoms (Lai et al., 2014). Alterations in serotonin concentration remains one of the most consistent biochemical findings within this diverse population, as many as 40-70% displaying increased platelet bound serotonin within general circulation (Hough & Segal, 2016; Whitaker-Azmitia, 2001). It has been proposed that increased circulating serotonin during critical periods of embryonic development, prior to complete formation of the blood-brain barrier, could overstimulate the central nervous system, altering typical developmental trajectories. This could induce negative feedback of the serotonergic system, stunting development and resulting in decreased neuronal serotonin, but chronically increased platelet bound serotonin concentrations throughout adulthood. These findings have appropriately translated into animal models, both those directly and indirectly induced with DHS, increased exposure to serotonin throughout fetal development. Many of these animals have been reported to display core features of the disorder such as an increase of stereotyped, repetitive behaviors and decreased sociability (Alzghoul et al., 2012; Kahne et al., 2002; Sprowles et al., 2016). Such studies validate the use of DHS induced Sprague Dawley rats as a model for ASD, as is used within this study.

Deficits in motor coordination, spanning both gross and fine motor function, are increasingly reported, with estimates of over 79% of affected individuals presenting with this dysfunction (Jeste, 2011; Lai et al., 2014). This study, in part, served to assess motor

coordination within DHS induced rats through implementation of a balance beam motor task. Our findings support reduced motor coordination in this animal model upon assessment using the large (28 mm) round beam (Figure 10A). The other two beams' trends suggested this, but did not reach significance with analysis (Figure 11A & 12A). Animals with alterations to the serotonergic system frequently display impairment of motor performance (Alzghoul et al., 2012; Ellegood et al., 2018; Tanaka et al., 2018). Therefore, while not entirely significant, our data remains encouraging of similar findings.

Individuals with ASD have also been observed to experience delays in motor learning, both explicit and implicit. This study aimed to assess implicit motor learning through analysis of Sprague Dawley rat improvement with repeated exposure to a balance beam motor task and implementation of an early motor training regimen for two of the four animal groups (Figure 4). We first established that all animals maintained the propensity for motor learning; all animals displayed improvement, reflected by decreased beam traverse time, with previous exposure to balance beam motor training (Figure 10B, 10C, 11B, 11C, 12B, & 12C). We then observed that, while not statistically significant, it appears animals induced with DHS displayed reduced improvement on the motor task with additional trials (Figure 10D, 10E, 11D, 11E, 12D, & 12E). This is suggestive that implicit motor learning remains intact, but compromised, within this animal model for ASD. Additionally, it alludes to the prospect of early motor training regimen implementation decreasing the severity of motor impairment as DHS induced animals, trained or untrained, all showed improvement with repeated trials (Figure 6, 7, & 8). Implementation of clinical motor training in human populations affected with ASD has proven to be promising, reports showing marked improvement on both explicitly taught and associated motor tasks (Travers et al., 2018).

Motor coordination and implicit motor learning are two processes highly reliant on modulation of PC excitation in response to external stimuli within the cerebellar cortex, providing a region of interest for further investigation. Humans with ASD frequently display alterations in cerebellar connectivity, specifically decreased glutamatergic excitation of PCs (Mostofsky et al., 2009). Additionally, serotonin is highly involved in cerebellar neurodevelopment, transient serotonin expression proving critical for appropriate dendritic elaboration, synapse formation and stabilization, and the stimulation of synaptic plasticity (Purves et al., 2001). This collectively suggests that alterations to the serotonergic system may alter cerebellar circuitry, reflected as impaired motor coordination and implicit motor learning. Therefore, we aimed to assess cerebellar circuitry, specifically connectivity between PCs and olivary climbing fibers.

Connectivity was assessed, indirectly, with immunohistochemistry and confocal microscopy of fluorescently tagged PCs and climbing fiber VGlut2. This staining was representative of glutamatergic PC, climbing fiber synapses with climbing fiber VGlut2 protein bound to PC glutamate receptors. The number of connections, the number of individual positively stained VGlut2 particles, as well as the volume of glutamate release and/or receptor abundance, the total area of positive VGlut2 staining, were quantified and analyzed. This revealed that animals induced with DHS display reduced synaptic connectivity and glutamate release and/or receptor expression (Figure 14A & 14B). Additionally, trained animals displayed increased synaptic connections and increased glutamate release and/or receptor expression. Increased cerebellar activation, glutamatergic release, has been alluded to be reflective of neuronal plasticity innate to motor learning (Mosconi et al., 2015). The formation of new synaptic connections, synaptogenesis, within the cerebellar cortex has also been suggested to be

representative of motor learning (Black et al., 1990). Therefore, our data suggests that the motor coordination and motor learning impairment displayed within rats induced with DHS is due to the cellular alterations within the cerebellum. The surplus of serotonin throughout development may alter cerebellar synaptic plasticity, inhibiting synaptogenesis and increased glutamatergic release in response to modulation from external stimuli. Additionally, our data suggests early motor training is sufficient to induce increased synaptogenesis and glutamate release within DHS induced animals. This supports the notion that early clinical intervention involving motor training may decrease motor deficit severity associated with ASD. With the established link between motor development and development of communicative abilities, motor training may be sufficient to lessen severity of symptoms associated with the disorder, even those within current diagnostic criteria.

Limitations to our current study included modest animal sample sizes and a potentially insufficient period of motor testing to definitively conclude all animals reached a plateaued, baseline motor performance. Both scenarios could be easily remedied if one desired to duplicate our findings. One could also limit the bars used for balance beam motor training and testing to the small (9.5) square and large (28 mm) round balance beams, for both appeared to be an appropriate level of difficulty, as reflected by animal failure rates (Figure 8, Table 1, & Table 2). In regards to our cerebellar circuitry findings, investigation of the upregulation of glutamate receptors on PCs is needed, perhaps through isolation and quantification of said receptors. This would assist in differentiating between increased volume of glutamate release and upregulation of receptor expression as an explanation of overall increased VGlut2 stained area. There could also be a large benefit to the implementation of supplementary behavioral tests, to not only further validate DHS induced rats as a model for ASD, but also to continue investigation of

potential effects of motor training regimens. Confirmation of a deficit in motor coordination and motor learning, as well as rescue of the deficit phenotype, could be further illustrated with the introduction of a rotarod or an adjustable ladder rung behavioral test. Furthermore, due to the communicative impact of motor dysfunction, it would be of particular interest to perform sociability assays to assess whether such is affected in DHS induced rats, as well as whether these may be rescued with motor training implementation, similarly to the study conducted in a brain hyperserotonemia mouse model by Tanaka, et al. (2018). One could also assess generalization of improved coordination by training animals on one motor task, then testing them on a different motor task. It would also be beneficial to further define circuitry alterations within the cerebellar cortex of DHS induced animals with the implementation of electrophysiological recordings of PCs; one could also assess if motor training contributes to the regulation of electrophysiological activity. Ultimately, there is much room for further study in this area of research to substantiate and further define our current findings.

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