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Cerebellar Dopaminergic Signaling in the Dorsomedial Striatum of Fragile-X Mice: Significance to Autism Spectrum Disorders

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CEREBELLAR DOPAMINERGIC SIGNALING IN THE DORSOMEDIAL STRIATUM OF FRAGILE-X MICE: SIGNIFICANCE TO AUTISM SPECTRUM DISORDERS

by

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Abstract

Autism spectrum disorders (ASD) are characterized by motor impairments and deficits in social communication potentially stemming from cerebellar Purkinje cell loss and dysfunction. Recent mapping of glutamatergic and dopaminergic pathways has provided evidence that degeneration of Purkinje cells coincides with attenuated dopamine (DA) release in the medial prefrontal cortex (mPFC) via the cerebello-thalamo-PFC pathway. As patients with ASD exhibit repetitive behavior, abnormal reward processing, and lack of planning ability, it appears the effects of neuropathology in the cerebellum extend to the nigrostriatal pathway. Using fixed-potential amperometry and electrical stimulation of cerebellar nuclei, we investigated the possibility that the cerebellum modulates DA release in the dorsomedial striatum, and additionally that this system is dysfunctional in rodent models of autism. Comparisons of Fragile-X mutant and wildtype mice revealed that the cerebellum directly modulates the nigrostriatal system in both strains, with no differences in functionality of DA signaling in the dorsomedial striatum.

Keywords: autism, dopamine, striatum, signaling, Fragile-X

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Cerebellar Dopaminergic Signaling in the Dorsomedial Striatum of Fragile-X Mice: Relevance to Autism Spectrum Disorders

The mammalian brain has evolved into an electrochemical network that functions to achieve the goals best suited for an organism's survival and to avoid those it deems most detrimental**.** All external and internal sensory information is processed and integrated, which contribute to a person's overall health and mental functioning in the environment. A disruption in this sensory integration is evident in people diagnosed with autism spectrum disorders (ASD). Patients with autism show deficits in recognizing common social cues in the environment, processing and retrieving items from internal memory, and implementing these aspects to plan and perform complex motor functions, which drive reward-seeking behavior.

Two major brain areas are involved in the coordination of sensory input and the development of motor processes: the cerebellum and the basal ganglia. The cerebellum—controller of the spatiotemporal aspects of movement—is pathologically developed in ASD with abnormal projections stemming to multiple nuclei throughout the brain, eventually producing attenuated neurotransmitter release in the frontal lobe. A subdivision of the major input station of the basal ganglia—the dorsomedial striatum—receives inputs from nearly all areas of the cerebral cortex to assemble goal-directed behavior, and is a likely candidate for dysfunction in autism. However, little research has examined if the cerebellum has modulatory connections with this region.

This research uses a systems neuroscience approach to investigate the notion that the cerebellum has projections to the nigrostriatal DA network, leading to cognitive and behavioral deficits in ASD through signaling dysfunction in the dorsomedial striatum.

Involvement of the Cerebellum in ASD

The specific etiology of autism remains unknown. However, investigations have linked genetic proclivities, viral infections, toxins, and developmental errors to the formulation of ASD (Rogers, Mckimm, et al., 2013). Each of these pathologies has emerged to produce a similar finding of changes among the substructure of the cerebellum. Within the cerebellum of the autistic brain, the most common neuropathology observed is loss of cortical Purkinje cells and hypoplasia (Bauman, 1991; Courchesne, Yeung-Courchesne, Hesselink, & Jerningan, 1988; Courchesne , Lincoln, Haas, & Schreibman, 1994; Courchesne, 1997; DiCicco-Bloom et al., 2006; Palmen, van Engeland, Hof, & Schmitz, 2004). These neurons appear to be necessary for sustaining coordination and homeostasis of the electrochemical communication throughout many systems in the brain.

Abnormal changes in the cerebellum or damage of these Purkinje cells commonly leads to a disruption in their profound signaling ability and can cause alterations in motor skills (Middleton & Strick, 2000; Thach, 1998), but how does this explain the cognitive deficits seen in autism? In ASD, level of motor skills has shown to be predictive of levels of autistic symptoms in later life (Sutera et al., 2007) suggesting an interrelation between motor and cognitive deficiencies. Modulation of cerebellar Purkinje cell output may serve as the starting point of this interrelation (Ciesielski & Knight, 1994), but locating affected downstream pathways and targets that modulate the motor and cognitive processes is an important link to understanding autism. Knowledge of these pathways may help to explain the deficits of memory, executive functioning and planning seen in patients with ASD. Comprehension of the Purkinje cell's systematic ability to process, encode, and

spread information throughout the brain is paramount for understanding the cerebellum's role in controlling the integration of these motor and cognitive signals. Essentially, it builds a foundation for intervention methods to diminish negative symptoms seen in ASD.

Information Processing in Purkinje Cells

Maintaining optimal functioning of Purkinje cells is proving to be critical for proper activity of many neural systems in the brain. The sensory association cortex and motor association cortex of the parietal and frontal lobes, respectively, have projections to the cerebellum that function to integrate and sharpen movement. These pathways send information to the mossy fibers (MF) of neurons in the pontine nuclei, which in turn relay inputs in a contralateral manner to the cerebellar cortex. These MFs form a small convergence of synapses on granule cells, which are the most abundant type of neuron in the human brain (Wechsler-Reva & Scott, 1999). Granule cells then recode information obtained from MFs and transmit a complete contextual account of MF activity through excitatory signals of parallel fibers, minimizing destructive interference and facilitating learning in Purkinje cells, which stimulates further signal output (Philipona & Oliver, 2004). Input from the cerebral cortex to the cerebellum is attributable to various types of nuclei (visual, spatial, premotor, motor), but complex synchronization of information allows Purkinje cells to provide the sole source of output from the cerebellar cortex, via activity on the deep cerebellar nuclei (Voogd & Glickstein, 1998).

Due to the GABAergic (γ-aminobutyric acid containing) nature of these neurons, Purkinje cells use inhibition to shape the spatiotemporal patterns of electrical and chemical signaling throughout the brain (Huang, 2007). Investigations of feed-forward

neural networks suggest that a single Purkinje cell can retain up to 40,000 input-output associations (Brunel, Hakim, Isope, Nadal, & Barbour, 2004). This multiplicity of interactions allows for continuous error recognition and correction of sensory stimuli, which produces fine-tuned movement best suited for the brain's current environment.

Cognitive and Behavioral Deficiencies in Autism

The frontal lobe is known to be involved in problem solving, executive functioning, self-awareness, and other aspects of social behavior (Chayer & Freedman, 2001). Patients with ASD exhibit many deficits in these areas of mental processing, and the frontal cortex of children with autism tends to be abnormally developed. Specifically, the medial and dorsolateral regions are sites of significant overgrowth when compared to controls (Carper & Courschesne, 2005), and when performing mental rotation tasks to assess competence in working memory and executive functioning, children with ASD revealed significantly less cortical activation in the prefrontal area (Silk et al., 2006). It is important to note that the degree of cerebellar abnormality in patients with autism is correlated with this increase in growth (Carper & Courschesne, 2005).

The studies above signify a presence of neural pathways connecting areas associated with motor functioning and cognitive processes. In regards to this assumption, Palesi et al. (2013) found that cerebellar hemispheres are connected via the ventrolateral thalamus with contralateral associative (prefrontal, parietal, temporal cortices) areas in the brain, supporting the notion that deficits in Purkinje cells may contribute simultaneously to malfunctions in motor skills and cognitive processes in ASD.

Recent in vivo neurochemical recordings of mice strains used to model autism show Purkinje cells regulate dopaminergic activity via projections from the DN to cognitive

centers in the brain and also exhibit a reorganization of mediating neuronal pathways. Mittleman, Goldowitz, Heck, & Blaha (2008) used DN electrical stimulation to evoke dopamine (DA) efflux in the medial prefrontal cortex (mPFC) of Lurcher mutant mice (a common model of ASD with 100% loss of Purkinje cells within the first 4 weeks of life) and compared their responses to wildtype mice that served as controls. The Lurcher mutants exhibited attenuation in mPFC DA release when compared to controls. This suggests that developmental loss of Purkinje cells, similar to that of ASD, can lead to a disruption in mPFC DA modulation. However, the specific nuclei involved in this disruption remain unclear.

Rogers, Dickson, et al. (2013) followed this pursuit by comparing cerebellar modulation of dopaminergic mPFC release in Lurchers and a mouse model of Fragile-X syndrome (FMR1 KO mice), which unveiled a reorganization of mediating neuronal pathways projecting to the mPFC. In this study, infusions of the sodium channel blocker lidocaine or the glutamate receptor antagonist kynurenate were used to inactivate dopaminergic and glutamatergic neuronal bodies (ventral tegmental area, thalamic mediodorsal, or thalamic ventrolateral), respectively, to compare functional adaptations of cerebello-cortico circuitry associated with abnormalities in cerebellar functioning. An attenuation of cerebellar-mPFC DA release in both mutant mice strains was found, along with a shift in strength of dopamine signal modulation towards the thalamic ventrolateral nuclei (ThN vl), away from the ventral tegmental pathway, while inactivation of the mediodorsal thalamic nuclei (ThN md) did not alter DA release significantly in either strain. A shift in modulatory strength towards the ThN vl is an important finding to note due to its known projections to the dorsomedial striatum (Jayaraman, 1985).

In addition to mutant mice strains showing neuronal pathology similar to those seen in autism, it is important to recognize that behavioral deficits in these mice also correlate with those seen in autism. Atypical behavior in children with autism may manifest in alterations of eating, aggression, and abnormal sleep patterns; with high exhibition of atypical behaviors negatively correlating with social skills and nonverbal IQ (Dominick, Davis, Lainhart, Tager-Flusberg, & Folstein, 2007). Repetitive hand and foot movements are also often seen in individuals diagnosed with ASD in addition to a reported lack of coordinated balance (Dowell, Mahone, & Mostofsky, 2009; Freitag, Kleser, Schneider, & von Gontard , 2007). As with most aspects of abnormal performance, a neurochemical issue is expected to prevail as a source of these deviations from typical behavioral functioning. Dickson et al. (2010) examined Lurcher chimeras, which have a variable loss of Purkinje cells, to determine if neuronal degradation has an effect on behavioral aspects of brain functioning. They found a negative correlation between executive functions, working memory, and repetitive behavior with the number of Purkinje cells. This suggests that the animal models used were efficacious in mimicking the symptomology seen in autism. However, for the current study we needed to ensure that the mouse strain used was the ideal candidate for assessing detriments in neural pathways. Although the Lurcher mutants were a potential choice, we chose a different strain which we believe will be more applicable.

Rodent Models of ASD

There are currently five genetic rodent models with neural and behavioral abnormalities coinciding with those seen in autism (Rogers et al., 2013). However, the FMR1 knockout mouse seems to be the most relevant to the current study. Fragile-X

syndrome is the most common monogenetic cause of autism, stemming from the silencing of the FMRP gene (Brown, 2005). FMR1 KO mice were designed to mimic the behavioral and neural symptoms of ASD such as elongated Purkinje cell spines, decreased cerebellar volume, learning deficiencies, and hyperactivity (Baker, 1994; Koeckoeck, 2005; Rogers et al., 2013). Although the Lurcher mutant mice have frequently been used to study autism, they have an autosomal dominant mutation which causes degeneration of all nearly all cerebellar Purkinje cells within the first few weeks of life (Vogel, Caston, Yuzaki, & Mariani, 2007). A complete loss of these cells does not adequately parallel the cerebellar pathology seen in autism. Thus, the FMR1 KO strain seems to be the ideal animal model for the current study.

Innovation

The combined use of behavioral and neurochemical experiments has provided evidence that mechanisms which govern detriments in motor skill learning and executive performance in autism arise, at least partially, from dysfunction of cerebellar manipulation on dopaminergic activity in the frontal lobe via the mesocortical dopaminergic pathway. Previously discussed research also provides ample evidence to initiate a search for neurochemical deficits in the nigrostriatal system, particularly the dorsomedial striatum; which we believe to be directly mediated by cerebellar efferents of the DN (Figure 1). With the knowledge of a shift in cerebellar modulatory strength toward the thalamic ventrolateral nuclei in rodent models of autism (Lurcher, FMR1) and the known connections between this nuclei and the striatum, it is expected that the dorsomedial striatum will exhibit abnormal DA release.

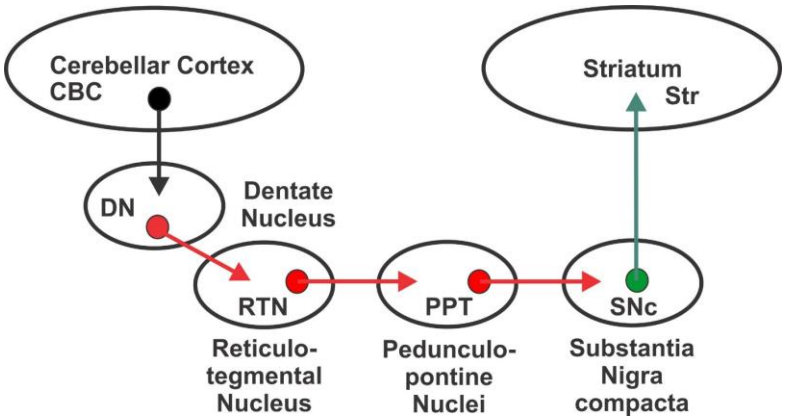

Figure 1. We predict that the cerebellum acts as a modulator of striatal dopamine release. This projected system occurs via polysynaptic inputs from cerebellar nuclei to dopamine-containing cells in the substantia nigra (SN), eventually leading to DA release in the dorsomedial striatum. Glutamatergic pathways are shown as red lines and the dopaminergic pathway as a green line.

Functioning of the Striatum

Modulation of the medium spiny neurons of the dorsal striatum allows for activation and deactivation of the direct and indirect pathways within the basal ganglia through activation of the expressed D1 and D2 receptors. The direct and indirect pathways enable the basal ganglia to interact with the motor cortex to select proper motor programs best suited for a person to gain rewards and simultaneously inhibit competing motor programs that are least beneficial in reward-seeking behavior, respectively (Kravitz & Kreitzer, 2012).

Within the motor and cognitive loops of the basal ganglia, the dorsomedial striatum acts not only as a subunit of the major input station, but also has developed connections to the associative cortex and many other neuronal sectors (hippocampus, amygdala, prefrontal cortex, thalamus); allowing for control of adaptive voluntary movement (Graybiel, Aosaki, Flaherty, & Kimura, 1994) and goal-directed actions, which are known to be involved in executive functioning (Da Cunha, Gomez, & Blaha,

2012). Understanding the functional relationship between cerebellar pathology and the dorsomedial striatum is pertinent to resolving symptoms seen in ASD.

Previous research has shown aberrant striatal functional connectivity with the anterior cingulate and frontal cortex, orbitofrontal cortex, and the brain stem (Di Martino et al., 2011). This may coincide with evidence of attenuated cerebellar modulation of DA release in the prefrontal cortex of Lurcher mutant and Fragile-X mice, along with alterations in modulatory DA control away from the VTA toward the thalamic pathway (Mittleman et al., 2008; Rogers et al., 2011, 2013). In regards to the dorsomedial striatum's involvement in pathway alterations and attenuated DA release in the mPFC, it is important to note that the striatum has connections with center median, ventrolateral and central lateral thalamic nuclei (Jayaraman, 1985). It is probable that these pathways contribute to many of the pathologies seen in autism. Evaluation of all evidence leads to our predicted hypothesis that mutant Fragile-X mice will show significant decreases in the DN-stimulation evoked dopamine response within the dorsomedial striatum in comparison to their wildtype controls.

Methods

Animals

Animals were bred and maintained at the University of Memphis in the Animal Care Facility located in the Department of Psychology. Mice were continuously maintained in a temperature-controlled environment (21±1 °C) on 12:12 light/dark cycle (lights on at 0800) and were given free access to food and water. All proposed experiments were approved by a local Institutional Animal Care and Use Committee and

conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Breeding

To produce Fragile-X experimental mice (FMR1) two phases of breeding were required. The first stage consists of male mice hemizygous for the FMR1^{tm1Cgr} targeted mutation (FVB. 129P2-FMR1^{tm1Cgr}/J, #004624) being mated with female wildtype control mice ((FVB.129P2-*Pde6b*$^{+}$ *Tyr*$^{c\text{-}ch}$/AntJ, #004828). The initial offspring produced litters composed of heterozygous females and wildtype males. The second stage consisted of heterozygous female mice being mated with wildtype male mice to produce litters containing both hemizygous and wildtype males. The wildtype littermates were used as control experimental subjects.

Surgery

A total of 15 subjects were used (9 FMR1 wildtype, 6 FMR1 mutant knockouts). All were urethane-anesthetized (1.5 g/kg i.p.) and placed in a stereotaxic frame. Body temperature was maintained at 36±0.5 °C with a temperature-regulated heating pad. Fixed potential amperometry (FPA) was used with a concentric bipolar stimulating electrode (SNE-100, Kopf Instruments), a carbon-fiber microelectrode (dopamine recording electrode; carbon fiber 10 µm o.d., 250 µm length, Thornel Type P, Union Carbide, Bristol, PA, USA), and an Ag/AgCl reference combination electrode. In individual mice, the stimulating and reference electrodes were implanted ipsilateral to one another in the right hemisphere, while the recording electrode was implanted contralateral in the left hemisphere; with respect to bregma and depth with respect to dura. Using stereotaxic coordinates in millimeter units, the stimulating electrode was

lowered into the DN of the cerebellum (AP = -6.25, ML = 2.1, DV = -2.4) and the DA recording electrode was implanted into the dorsomedial striatum (AP = 1.5, ML = .8, DV = 2.8).

Fixed-potential Amperometry

FPA coupled with carbon-fiber dopamine recording microelectrodes is a technique for real-time monitoring of stimulation-evoked dopamine release. Following implantation of all electrodes, a constant voltage of +0.8 V was applied to the recording electrode, and an oxidation current, reflective of dopamine concentrations, was sampled continuously (10,000 samples/s) via an electrometer (ED401 e-corder 401 and EA162 Picostat, eDAQ Inc., Colorado Springs, CO, USA) filtered at 10 Hz low pass. A total of 100 stimulations (monophasic 0.5 ms duration pulses at 50 Hz every 60 s) was applied to the DN (at 800 µAmps) via the stimulating electrode with use of an optical isolator and programmable pulse generator (Iso-Flex/Master-8; AMPI, Jerusalem, Israel). As seen in Figure 2, the recording electrode was placed in the dorsomedial striatum to monitor DA concentration.

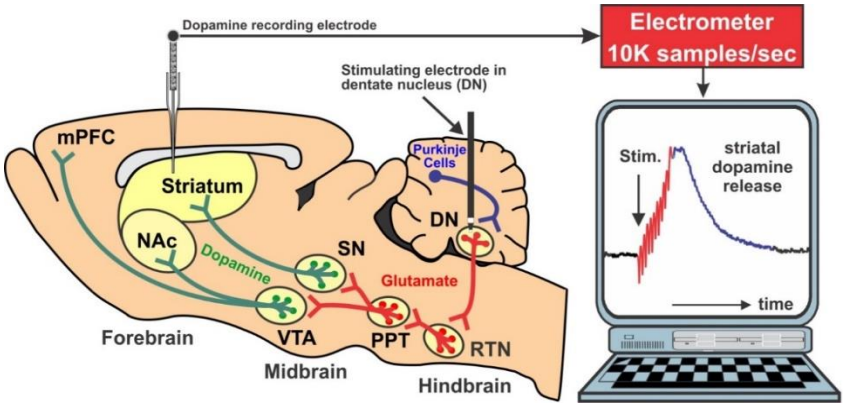

Figure 2. A stimulating electrode was placed in the deep cerebellar nuclei (dentate nucleus) and a carbon-fiber recording electrode monitered dopamine release in the dorsomedial striatum.

Histology

Immediately following each experiment, a direct current (100 µA for 10s; +5 V for 5 s) was passed through the stimulating electrode in the DN and through the recording electrode in the dorsomedial striatum to lesion tissue in each site. Each mouse was euthanized with a lethal intracardial injection of urethane. Brains were removed and preserved overnight in 10% buffered formalin containing 0.1 % potassium ferricyanide, and then stored in 30 % sucrose/10 % formalin solution until sectioning. Brains were sectioned on a cryostat at -30℃. A Prussian blue spot indicative of the redox reaction of ferricyanide and iron deposits labels the stimulating electrode in the dentate, and the location of the recording probe was determined by the electrolytic lesion.

Data Analysis

DN stimulation-evoked was extracted from the amperometric current recordings within the range of 0.2 s to 30 s post stimulation (-0.2 s through 30 s) for each of the mutant and wildtype mice. The percent difference in overall magnitude of DA release in the dorsomedial striatum was summed and compared for each group (KO, WT) using a between-groups analysis of variance (ANOVA). Average magnitude of DN-evoked dopamine oxidation current was the dependent variable and the independent variable was the mouse strain (KO, WT).

Results

A total of 15 mice, 9 WT and 6 KO, were used in our analysis. Responses to electrical stimulation were obtained 2 seconds pre-stimulation to 30 seconds post-stimulation (Figure 3), and used to calculate an average DA concentration value (Figure 4) in micromoles (µM) using flow injection analysis data and FPA data for both the

FMR1 wildtypes (*M* = 0.002063, *SD* = 0.000959, *SEM* = 0.0003196) and the FMR1 KO groups (*M* = 0.00183, *SD* = 0.002734, *SEM* = 0.0011161). To assess this, a one-way between subjects ANOVA was used and indicated that there was not a significant difference between FMR1 mutant mice and wildtypes when comparing magnitude of DA release in the dorsomedial striatum at the p<.05 level [*F*(1, 13) = 4.67, p = 0.83]. These results suggest that the cerebellum does act to modulate DA signaling in the nigrostriatal pathway and that the neural pathologies seen in the FMR1 mutant mice do not extend to the dorsomedial striatum in terms of DA release. However, it should be noted that downstream output of the dorsomedial striatum was not assessed and its functioning could be of question.

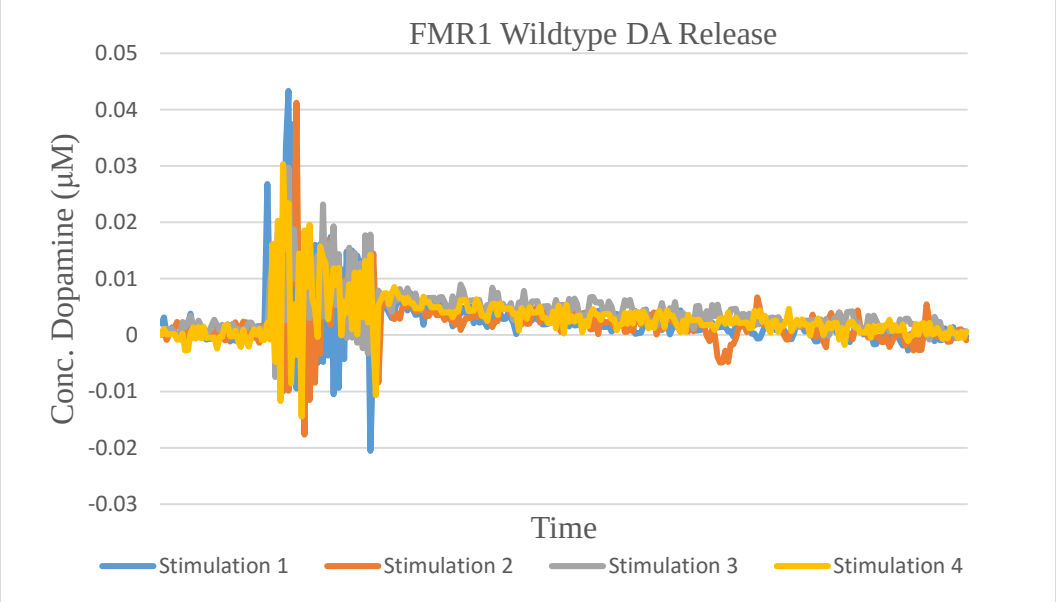

Figure 3. Recorded magnitude of DA release in FMR1 wildtype 2 seconds pre-stimulation to 30 seconds post-stimulation. Concentration of neurotransmitter is shown in micromoles (µM).

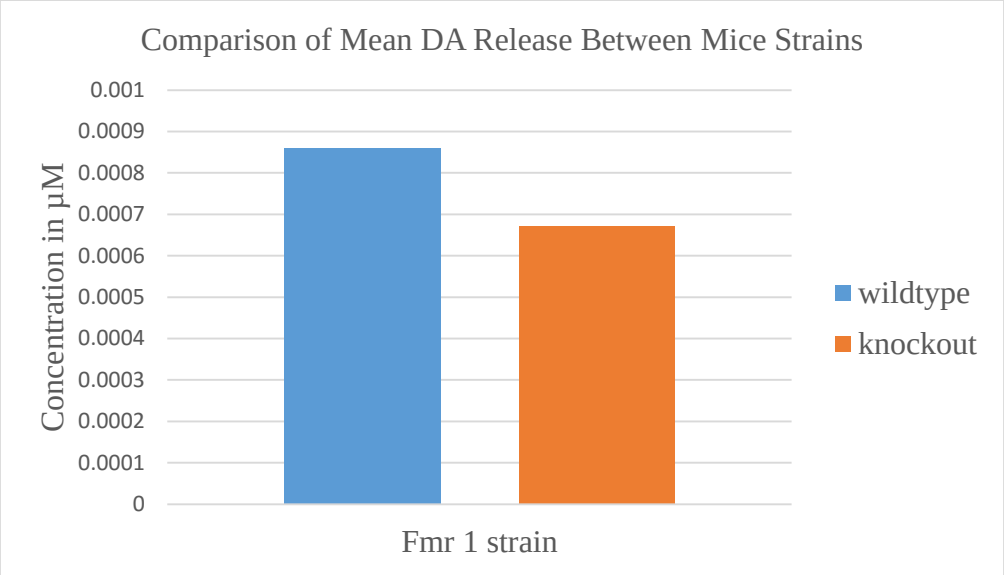

Figure 4. Average stimulation-evoked dopamine release in Fragile-X mutants (*M* = 0.00183 µM) and wildtypes (*M* = 0.002063 µM) in micromoles. No significant differences were found between groups.

Discussion

The aim of the current study was to determine if the cerebellum modulates the nigrostriatal dopamine pathway in the mammalian brain, and if so, to understand if dopaminergic transmission in the dorsomedial striatum is abnormal in ASD. Our results support the notion of cerebellar modulation on the nigrostriatal pathway due to both mice strains (WT, KO) exhibiting DA release when stimulated. However, we found no significant difference in this dopaminergic release within the dorsomedial striatum between our mutant and control mice. These results suggest that the dorsomedial striatum is functional, but further investigation is necessary to arrive at a conclusive statement on the meaning of these findings.

It is imperative that other rodent models of autism, particularly Lurcher mutants, be examined to determine the functionality of the dorsomedial striatum. Rogers et al.

(2013) has shown that both Lurcher and FMR1 mutant mice show attenuations in cerebellar stimulation-evoked DA release in the mPFC with an accompanying reorganization of neuronal pathways. A comparative analysis of a mouse strain lacking all Purkinje cells (Lurcher) and a strain modeling Fragile-X syndrome (reduced number/maldeveloped Purkinje cells) would be highly beneficial to our understanding of the striatum's role in this disorder (Mittleman et al., 2008). If the dorsomedial striatum is found to be functional in Lurchers as well, we could then begin to search for deficits in downstream pathways and surrounding nuclei to pinpoint likely disruptions. It is possible that other nuclei within the basal ganglia circuitry could be subject to dysregulation. If it is found that Lurcher mutants exhibit abnormal DA release in the dorsomedial striatum when compared to controls, one must search to understand how these abnormally developed Purkinje cells in FMR1 mice are still able to signal properly. With the brain's highly plastic nature, this is a possibility and should be examined.

Additionally, this experiment sought to obtain levels of DA release, but had no assessment of neurotransmitter binding efficacy or receptor activation. The D1 and D2 G-protein coupled receptors (D1DR, D2DR) located within the striatum are complex proteins that are dependent upon spatiotemporal signaling. These receptors play a major role in the inhibition network, which has shown to be deficient in neurodegenerative diseases such as schizophrenia, addiction, and Parkinson's (Barnett et al., 2010; Gauggel, Rieger, & Feghoff, 2003; Koob & Volkow, 2010). When this inhibition network is examined in individuals with high-functioning autism, the brain areas involved show decreased activation and under-connectivity (Kana, Keller, Minshew, & Just, 2007). Specifically, when individuals were asked to complete a response-inhibition task they

showed lower levels of synchronization within the inhibition network (anterior cingulate gyrus, middle cingulate gyrus, and insula) and the right middle and inferior frontal and right inferior parietal regions. Eagle et al. (2011) used a stop-signal task and D1/D2 receptor antagonists to examine the role of the dorsomedial striatum and nucleus accumbens core in behavioral inhibition; finding that receptors in the striatum, but not the nucleus accumbens core act to balance behavioral inhibition. In order to further assess the role of the dorsomedial striatum in this disorder, FMR1 mutant mice should be tested on a behavioral inhibition measure similar to the stop-signal task. Di Martino et al. (2011) found that the striatum has connections branching to each of the areas mentioned (cingulate cortex, insula, parietal cortex), and in autism, has developed connectivity with other areas not seen in typically developing children. Interestingly, it was found that the striatum shows hyperconnectivity to the pons and insula. If FMR1 mutant mice show decreased behavioral inhibition when compared to controls, it may help develop an understanding of how the D1DR and D2DR function in ASD.

The autism disconnection hypothesis has mostly been supported by findings of decreased function of corticocortical networks, but it is becoming clear that subcortical nuclei are a major determining factor in some of the symptoms seen in ASD. With its extensive connections throughout the brain, the dorsomedial striatum may act as an intermediary waypoint which contributes much of the lowered connectivity between the subcortical and cortical nuclei. Although the current study showed no significant findings of DA release in this area, future behavioral assessment of rodent ASD models and examination of other basal ganglia circuitry (nucleus accumbens, substantia nigra,

dorsolateral striatum) may reveal neural abnormalities significant to resolving degenerative symptoms seen in ASD.

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