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Examining Cerebellar Modulation of Mesolimbic Dopamine Transmission Using Fixed Potential Amperometry

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EXAMINING CEREBELLAR MODULATION OF MESOLIMBIC DOPAMINE TRANSMISSION USING FIXED POTENTIAL AMPEROMETRY

by

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Dedication

I dedicate the many experiments and completion of this dissertation to Silsila. Without her support and guidance, I would not have finished the turbulence of graduate school. From the bottom of my soul, thank you.

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Preface

Dopamine is a multifunctional molecule known to be involved in many neural processes including reward, executive functions, and sensorimotor processing (Schultz, 2010). Dopamine operates at the synapse on the order of milliseconds, and the magnitude and timing of phasic release is critical for understanding how variation in release can relate to behavioral functions or pathology (Tsai et al., 2009; Venton et al., 2003). Fixed-potential amperometry (FPA) combined with carbon fiber microelectrodes has proven to be effective at probing dopaminergic microsystems and precisely describing the kinetic mechanisms that regulate the phasic response (Fathali & Cans, 2018; Lester, Rogers, & Blaha, 2010; Mosharov & Sulzer, 2005). Chapter 1 includes applications of *in vivo* fixed potential amperometry (FPA) in exploring normal and pathological function of dopaminergic circuitry and is followed by two separate manuscripts. The first study (Chapter 2) has been formatted for submission to the journal Synapse and provides a systematic examination of phasic transmission in the four predominant output regions of the nigrostriatal and mesocorticolimbic pathways using FPA in anesthetized mice. Chapter 3 is formatted according to APA guidelines and presents data on the use of amperometry for investigating dopamine dysfunction in a mouse model of autism as well as assessing the cerebellar role in modulating the nigrostriatal dopamine system. Chapter 4 focuses on cerebellar-mediated asymmetry in the mesolimbic dopamine system through examining projections from the cerebellar dentate (DN) to the nucleus accumbens and has been formatted according to APA guidelines. The final chapter (Chapter 5) provides an overall summary of the amperometric experiments and discusses the implications of the results.

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Abstract

Holloway, Z. R., Ph. D. The University of Memphis. December, 2018. Examining Cerebellar Modulation of Mesolimbic Dopamine Transmission Using Fixed Potential Amperometry. Major Professors: Deranda B. Lester, Ph.D. and Helen J. Sable, Ph. D.

Elucidating how dopamine neurons operate regularly in aspects of neurochemical release and in pathological systems is essential for understanding their role in behavior, drug treatment, and disease. Fixed-potential amperometry (FPA) combined with carbon fiber microelectrodes has proven to be effective at probing these dynamic microsystems and precisely describing the regulatory mechanisms that govern dopamine neurotransmission. To date, we have utilized FPA to: a) observe and quantify differences in the neurochemical profile of phasic dopamine release in major dopaminergic afferents including the striatum, nucleus accumbens (NAc), amygdala, and prefrontal cortex through stimulation of the medial forebrain bundle (MFB), b) provide evidence that differing behavioral processes in the brain emerge from spatial and temporal variations in the phasic response, and c) identify a pathway originating in the cerebellar dentate that projects to nigrostriatal and mesolimbic systems, solidifying the role of the cerebellum in higher cognitive functions and neuropathology related to dopamine dysfunction. These findings provide evidence that the cerebellum regulates dopamine release in the cerebrum, and previous literature has shown that dopaminergic systems in the bilateral cerebral hemispheres contribute asymmetrically to behavior, structure, and function. To determine whether asymmetrical lateralization in the dopaminergic system occurs at the level of the cerebellum, cerebrum, or both, FPA was used to examine asymmetry of dopamine release in the dentate nucleus (DN)-NAc and MFB-NAc pathways. We found significant differences in the amplitude of phasic dopamine release in the DN-NAc systems, but not the MFB-NAc pathways. Results from this study support the notion that reward processes in the brain may be lateralized between cerebrocerebellar networks, with greater phasic release occurring in projections from the left cerebellar DN to the right NAc. These studies may provide more detailed information about the relationship between the cerebrocerebellar networks and lateralization of the dopaminergic system as well as potentially reveal novel targets for pharmacological interventions in neuropathology of the cerebellum.

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Abbreviations

Analysis of Variance (ANOVA)

Attention Deficit Hyperactivity Disorder (ADHD)

Autism Spectrum Disorders (ASD)

Basolateral Amygdala (BLA)

Dentate Nucleus (DN)

Dopamine (DA)

Dopamine Transporter (DAT)

Fast Scan Cyclic Voltammetry (FSCV)

Fixed Potential Amperometry (FPA)

Intraperitoneal (ip)

Knockout (KO)

Medial Prefrontal Cortex (mPFC)

Medial Forebrain Bundle (MFB)

Mediodorsal Thalamic Nuclei (ThN md)

Mossy Fibers (MF)

Norepinephrine Transporter (NET)

Nucleus Accumbens (NAc)

Prefrontal Cortex (PFC)

Purkinje Cells (PK cells)

Substantia Nigra Pars Compacta (SNc)

Test Stimulation 1 (T1)

Test Stimulation 2 (T2)

Thalamic Ventrolateral Nuclei (ThN vl)

Ventral Tegmental Area (VTA)

Wildtype (WT)

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CHAPTER 1: ELECTROCHEMICAL DETECTION OF DOPAMINE DYNAMICS

Introduction

Basal Versus Phasic Dopamine Release

The nigrostriatal dopamine system consists of dopamine cell bodies in the substantia nigra pars compacta (SNc) that project to the dorsal striatum and play a role in the expression of motor processes (Horvitz, 2000; Parent & Hazrati, 1995). The other major dopaminergic circuit, the mesocorticolimbic dopamine system, consists of cell bodies in the ventral tegmental area (VTA) that project to the amygdala, nucleus accumbens (NAc), and medial prefrontal cortex (mPFC) (Fibiger & Phillips, 1988; Koob & Swerdlow, 1988). Rather than motor acts, the purpose of these structures is to fine-tune aversive, reward, and cognitive processes, respectively (Davis, 1992; Lee, Lee, & Kim, 2017; Matsumoto & Hikosaka, 2009; Seamans & Yang, 2004). Dopamine neurons mediate behavior by using two bursting modes. Basal dopamine is generated when neurons are tonically active at slow rates (~4 Hz), producing low concentrations of extracellular dopamine; this occurs when no behaviorally relevant stimuli are present (Goto, Otani, & Grace, 2007). Conversely, when presented with sensory signals predicting rewards or aversive events, these cell bodies fire phasically at high frequencies and serve as a learning signal (Schultz, Apicella, & Ljungberg, 1993; Venton et al., 2005). Phasic release occurs in response to salient information and leads to elevated levels of extracellular dopamine, and this transient rise can influence motor output in behavioral situations (Phillips et al. 2003; Robinson et al. 2002). Dopamine operates at the synapse on the order of milliseconds, and the magnitude and timing of phasic release is critical for many diverse functions including reward, attention, anxiety, and decision-

making (Tsai et al., 2009.) These differing behavioral processes emerge from variations in autoreceptor, uptake, and release sites, which lead to site-specific differences in dopamine concentrations during synchronous phasic firing (Venton et al., 2003). Quantitative predictions of how the phasic dopamine response operates in major midbrain afferents of the mesocorticolimbic and nigrostriatal systems are crucial for understanding the influence of dopamine on behavior and for the development efficient treatments for disorders related to dopamine dysfunction.

Electrochemical Measures of Dopamine

The ability to electrochemically measure dopamine release and properties related to dopamine kinetics during pharmacological treatments and behavioral tests has greatly advanced the study of dopaminergic neurotransmission. Analysis of many vital dopaminergic events in the brain requires an electrochemical technique with superb filtering, signal-to-noise ratio, sampling rate, and chemical selectivity (Michael & Wightman, 1999). Although techniques like microdialysis have been used to monitor slow or tonic alterations in neurotransmitter levels spanning minutes (Borland et al. 2005), the rapid chemical changes that occur during phasic release require a more suitable approach. Fast-scan cyclic voltammetry (FSCV) is a popular method used to measure subsecond dopamine efflux and has excellent chemical selectivity with a high sampling rate (10 times per second) (Garris & Wightman, 1994). However, analysis of the phasic response in the synaptic space requires a technique with an instantaneous response to DA release.

In vivo fixed-potential amperometry (FPA) offers the best temporal resolution available (10,000 samples per second) and previous studies have shown FPA coupled

with carbon-fiber DA recording microelectrodes to be a valid technique for real-time monitoring of stimulation-evoked DA release (Agnesi, Blaha, Lin & Lee, 2010; Agnesi et al., 2009; Forster & Blaha, 2003).

The high sampling rate of FPA permits the quantification of the transmitter molecules released from individual vesicles, which provides valuable information about the real-time kinetics and homeostatic mechanisms involved in dopamine release during exocytosis (Mosharov & Sulzer, 2005). However, amperometry has poor chemical selectivity because other monoamines such as serotonin and norepinephrine are also oxidized at +0.8 V. Therefore, after baseline dopamine release has been established, animals can be injected systemically with either saline as a control, a dopamine uptake blocker, a serotonin uptake blocker, or a norepinephrine uptake blocker to validate that dopamine was the oxidized molecule being monitored in the experiments. Thirty minutes after injection is considered to be efficient time for the drug to be absorbed and if dopamine is the molecule being recorded, only the dopamine uptake blocker will elevate extracellular levels (Mittleman et al., 2011; Tye, Miller, & Blaha, 2013). Saline along with the other uptake blockers cause no significant changes in dopamine oxidation current due to their molecular properties and binding sites. The flexibility of this electrochemical approach not only allows for quantification of DA release with excellent signal-to-noise ratio, but also for additional aspects of phasic transmission such as uptake, autoreceptor sensitivity, overall dopamine supply, and changes in dopamine half-life following various uptake blockers (Fielding et al., 2013; Lester, Rogers, & Blaha, 2010; Mittleman et al., 2011).

Electrochemistry of Fixed Potential Amperometry

Electrochemical detection of release of oxidizable neurotransmitters in FPA requires a three-electrode configuration that incorporates an auxiliary electrode, reference electrode, and a recording electrode, and recordings are performed within a Faraday cage to increase the signal to noise ratio and eliminate static interference (Blaha & Phillips, 1996; Forester & Blaha, 2003). An electrometer and analog to digital chart recorder form a circuit between the three electrodes, and a fixed continuous potential (+0.8 V) is applied to the recording electrode via the auxiliary electrode, while maintaining a potential difference between the recording and reference electrode (Blaha and Phillips, 1992). By applying a positive potential to the carbon fiber recording electrode, exocytotic activity from dopamine neurons is observed as amperometric spikes, referred to as oxidation current, which is caused by the transfer of electrons after monamine oxidation (Mosharov & Sulzer, 2005). The positive potential of the electrode is constant, so dopamine oxidation current is always directly proportional to the concentration of dopamine (Michael & Wightman, 1999). Responses become smaller and wider as diffusional distance of the neurotransmitter concentration from the recording electrode increases (Chow & von Ruden, 1995; Evanko, 2005). The amplitude and duration of spikes give notion to the quantal size of the release event (Bruns, 2004). Dopamine can act locally at receptors adjacent to release sites or diffuse further to remote receptors, and the characteristics of individual spikes provide information about how dopamine signaling varies in distinct neural systems with assorted behavioral functions (Mosharov & Sulzer, 2005).

Measuring Stimulated Dopamine Release

Modeling a particular neural region in mice requires stereotaxic coordinates for the system and can be found in relation to bregma, midline, and dura in the mouse atlas of Paxinos and Franklin (2001). In each mouse, a concentric bipolar stimulating electrode is typically implanted at the origin of the nigrostriatal and mesocorticolimbic dopaminergic circuitry in midbrain, or the bundle of axons known as the medial forebrain bundle (MFB) extending from these cell bodies. Stimulation of the MFB has been shown to evoke dopamine release in all four major terminal regions (Garris & Wightman, 1994; Jones, Harris, Kilts, & Wightman 1995).

The FPA set-up also requires a stainless-steel auxiliary and Ag/AgCl reference electrode combination is placed on the surface of contralateral cortical tissue, and a carbon fiber recording electrode is positioned in the synaptic space near a secretory cell of interest. Stimulation protocols vary between experiments, but often consist of short monophasic pulses (400-800 μAmps) at 20-50 Hz to establish a baseline dopamine response. The amplitude and duration of the of the response observed immediately after stimulation is representative of the dopamine oxidation current in the synaptic space of the observed area and is typically converted into dopamine concentration. This conversion typically requires a flow injection system and in vitro calibration of the recording electrodes in dopamine solutions (0.2-1.2 μM) after recording electrodes have been removed from the brain (Michael & Wightman, 1999). Change in dopamine oxidation current (nAmp) can then be converted to dopamine concentration (μM). FPA allows for a systematic quantification of DA release, and can also be used to examine

mechanisms that regulate the dopaminergic profile, such as autoreceptor functioning, dopamine supply levels, and the dopamine transporter.

Dopamine Autoreceptor Functioning

Presynaptic inhibition allows for neurons to modulate their own neurotransmitter release through the function of neurotransmitter-specific autoreceptors (Stark et al., 1989). In vivo, the onset of D2 inhibition is maximal between 150 and 300 ms after the end of the initial conditioning stimulation (Benoit-Marnand et al., 2001). The sensitivity autoreceptors can be assessed with amperometry by using five sets of conditioning pulses or pre-pulses (1, 5, 10, 20, and 40 pp), delivered 300ms prior to the second test stimulation (T2), in which T2 has a stimulation protocol similar to that of recording stimulation-evoked baseline release. T2 can then be compared to an identical prior stimulation (T1) which does not follow pre-pulses. Pre-pulses before test stimulations evoke enough dopamine to bind to D2 receptors and activate the autoreceptor function. By increasing the amount of pre-pulses prior to stimulation it is possible to observe various levels of autoreceptor activation and efficacy. Low-to-high autoreceptor sensitivity is indicated by low-to-high percent inhibition of evoked dopamine efflux, such that high sensitivity would result in a lower amplitude of the stimulation-event with pre-pulses (T2) compared to the event with no pre-pulses (T1).

Presynaptic Dopamine Depletion

Each dopamine terminal has a reserve of neurotransmitters available for release when challenged environmentally or experimentally, via pharmaceuticals or electrical stimulation (Pothos, Davila, & Sulzer, 1998; Willuhn, Burgeno, Groblewski, & Phillips, 2014). Assessment of the overall dopamine supply level can be experimentally tested

with amperometry in output modules of the nigrostriatal and mesocorticolimbic system using a continuous 3 min stimulation (20-50hz) to completely deplete neuronal reserves (Fielding et al., 2013). Functional differences in reserves of these modules may provide useful insight to the application of medications which rapidly elevate the extracellular concentration of dopamine in the brain, such as amphetamine formulations in treatments for ADHD (James et al., 2001; Kahlig & Galli 2003).

Presynaptic Dopamine Transporters

After obtaining baseline recordings of dopamine efflux with amperometry, dopamine uptake can be quantified by measuring dopamine half-life decay, which is the time for 50% decrease from the maximum evoked amplitude to the pre-stimulation baseline level. Amperometric measurements of half-life decay are an indicator of DAT functioning and allow for pharmaceutical challenges including reuptake blockade (Benoit-Marand, Jaber, & Gonon, 2000; Mittleman et al., 2011). Analysis of various reuptake blockers within a dopaminergic system can also be used to validate that dopamine was the oxidized molecule being monitored in the experiments.

Summary and Conclusions

Amperometry is a powerful analytical tool and is one of the few methods providing quantitative information about single-vesicle neurotransmitter release (Mosharov & Sulzer, 2005; Wightman et al., 1991). The relative simplicity of its design and experimental set-up equip the researcher with the capability to examine any neural system or model of neuropathological illness, and the temporal resolution of this technique (10,000 sample/sec) is higher than any current electrochemical method. Amperometric techniques combined with carbon fiber microelectrodes have confirmed

their effectiveness at probing these ultra-small systems and precisely describing the regulatory mechanisms that govern dopamine neurotransmission. Elucidating how dopamine neurons operate regularly in aspects of neurochemical release and in pathological systems is essential for interpreting their respective roles in behavior, drug treatment, and disease, and amperometry provides a especially useful tool to explore uncharted dopaminergic territory.

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CHAPTER 2: COMPARING PHASIC DOPAMINE DYNAMICS IN THE STRIATUM, NUCLEUS ACCUMBENS, AMYGDALA, AND MEDIAL PREFRONTAL CORTEX

Introduction

Neural modules involved in emotion, reward, executive functions, and action-selection are all regulated by the same chemical signal, bursts of dopamine (DA), which originate from neuronal firing deep within the midbrain (Schultz, 2010). Dopaminergic axons from the midbrain are distributed to multiple brain regions in two independent, parallel circuits—the nigrostriatal and mesocorticolimbic dopamine pathways—each projecting to many highly interconnected modules of the basal ganglia, limbic system, and frontal cortex. The modules and their interconnecting feedback networks make up larger systems termed the motor, motivational, and associative corticostriatal loops, which are independent neural networks reciprocally connecting the basal ganglia and other subcortical nuclei with the cerebral cortex (McHaffie, Stanford, Stein, Coizet, & Redgrave, 2005; Seger, 2009). Dopamine has a major role in regulating the activity of these loops (Voorn, Vanderschuren, Groenewegen, Robbins, & Pennartz, 2004; Haber, 2014; Haber, Kim, Mailly, & Calzavara, 2006).

The nigrostriatal dopamine system consists of dopamine cell bodies in the substantia nigra pars compacta (SNc) that project to the dorsal striatum, a prominent part of the motor loop (Parent & Hazrati, 1995). Dopamine in the striatum is necessary for the initiation of voluntary movement, and dysregulation of the nigrostriatal dopamine system can result in severe deficits in movement initiation and execution, such as the cardinal motor symptoms of Parkinson's disease (Lewis, Slabosz, Robbins, Barker, & Owen,

2005; Schultz et al.,1989). Dopamine receiving nuclei in the mesocorticolimbic and nigrostriatal pathways are distinguished by their unique roles in behavior, but it remains unclear how underlying dopamine transmission varies in these different regions.

The mesocorticolimbic dopamine system consists of cell bodies in the ventral tegmental area (VTA) that project to the amygdala, nucleus accumbens (NAc), and medial prefrontal cortex (mPFC) (Fibiger & Phillips, 1988; Koob & Swerdlow, 1988). These systems are an integral part of the motivational loop, in which the medial network of the frontal cortex projects not only to the nucleus accumbens, but also the striatum which receives inputs from the amygdala (Ikemoto, Yang, & Tan, 2015). Dopamine in the amygdala is important for recognition and regulation of fear memories (Lee, Lee, & Kim, 2017), and pathology within this system is associated with anxiety disorders (Davis, 1992). Dopamine release in the NAc codes for reinforcement of internal and external rewards, and electrophysiological recordings of dopamine cell bodies in the VTA have shown these cells increase their firing rates in response to the presentation of salient, rewarding stimuli and decrease firing in response to negative stimuli (Cohen, Haesler, Vong, Lowell, & Uchida, 2012; Matsumoto & Hikosaka, 2009; Mirenowicz & Schultz, 1996; Robinson, Howard, McConnell, Gonzales, & Wightman, 2009). Most drugs of abuse increase extracellular NAc dopamine levels, which can lead to addiction (Di Chiara, 2002; Russo et al., 2010). Addiction has been described as the pathology of motivation, in which the entire pathway of goal-directed behavior undergoes pathological modification (Kalivas & Volkow, 2005). Electrophysiological recordings indicate that dopamine neurons innervating the mPFC are functionally distinct from those projecting to the NAc, therefore differing behavioral functions are to be expected as well (Bannon &

Roth, 1983). Dopamine in the mPFC is thought to influence many cognitive functions that support associative learning, such as working memory, planning, and attention; furthermore, dysfunctions in mPFC dopamine may underlie both positive and negative symptoms and cognitive deficits associated with schizophrenia (Popescu, Zhou, & Poo, 2016; Seamans & Yang, 2004).

In freely moving rats, dopamine neurons fire tonically at ~4hz and burst fire phasically at ~20hz (Hyland et al., 2002). Tonic firing is suggested to occur when no behaviorally relevant stimuli are present and produces low concentrations of extracellular dopamine (Goto, Otani, & Grace, 2007). Conversely, it has been suggested that phasic firing occurs in relation to behaviorally significant external stimuli whose detection is crucial for learning (Schultz, Apicella, & Ljungberg, 1993). This mode of firing is thought to evoke a large enough extracellular concentration for the highlighting of salient stimuli in the environment (Middleton and Strick, 2000). In an experimental setting, the frequency with which these neurons are electrically stimulated determines their effects on release; 25 pulses at 50hz elevates the extracellular concentration sufficient for reward, but 25 pulses at 1hz does not due to the fast action of the uptake system (Bass et al., 2010; Ikemoto, Yang, & Tan, 2015; Tsai et al., 2009). The multifunctionality of dopamine, juggling aspects of behavior such as action-selection, emotion, and motivation likely lie at the individual synapses, with varied amount of phasic dopamine release acting on different receptor populations.

Previous studies have shown regional differences in phasic dopamine transmission. Although release concentrations were found to be similar across regions, the rate of dopamine uptake was slower in the amygdala and mPFC compared to the NAc

and striatum (Garris & Wightman, 1994). Furthermore, these areas have previously been found to respond differently to dopamine agonists. Dopamine uptake blockers, such as cocaine and nomifensine, had no effect in the amygdala but greatly increased extracellular dopamine concentrations in the NAc and striatum (Jones, Garris, Kilts, & Wightman, 1995). It should be noted that these studies were conducted using fast-scan cyclic voltammetry (FSCV) in brain slices. FSCV has excellent chemical selectivity and can accurately quantify sub-second dopamine efflux, sampling every 100ms (10 times per second) (Garris & Wightman, 1994). However, in vivo FPA has the best temporal resolution available (10,000 samples per second) and is more suitable for documenting rapid dopaminergic events at the synapse. Thus, we employed this technique in the current study.

Given that chemical selectivity is lacking with fixed potential amperometry, we pharmaceutically confirmed that our recordings are dopamine-dependent using uptake blockers targeting various neurotransmitters. With this electrochemical technique, it is possible not only to quantify dopamine release and uptake (an indication of dopamine transporter [DAT] functioning) with excellent signal to noise ratio, but also examine additional aspects of phasic dopamine transmission, such as dopamine autoreceptor sensitivity, overall dopamine supply, and changes in dopamine half-life following the uptake blockers (Fielding et al., 2013; Lester, Rogers, & Blaha, 2010; Mittleman et al., 2011). This study provides a comprehensive, systematic examination of phasic dopamine transmission in the 4 predominant neural output regions of the nigrostriatal and mesocorticolimbic pathways using fixed potential amperometry in anesthetized mice. Many drugs, both therapeutic and recreational, alter dopamine levels; thus, an

understanding of regional differences in dopamine transmission can lead to increased knowledge about the influence of dopamine on behavior and more efficient treatments for disorders related to dopamine dysfunction.

Materials and Methods

Animals

Fifty-six male C57BL/6J mice (Jackson Laboratories, ME) were housed 3-5 per cage in a temperature-controlled environment (21±1 °C) on a 12 hr light/dark cycle with (lights on at 0600) and given food and water available *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Memphis and conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Efforts were made to reduce the number of animals used and to minimize pain and discomfort.

Surgery

Mice were anesthetized with urethane (1.5 g/kg, i.p.) and placed in a stereotaxic frame (David Kopf Instruments, Tujundga, CA) ensuring flat skull. Body temperature was maintained at 36 ± 0.5 °C with a temperature-regulated heating pad (TC-1000; CWE, NY). All stereotaxic coordinates are in mm from bregma, midline, and dura according to the mouse atlas of Paxinos and Franklin (2001). In each mouse, a concentric bipolar stimulating electrode (SNE-100, Rhodes Medical, CA) was implanted into the left medial forebrain bundle (MFB) (AP -2.0, ML +1.1, and DV -4.0), which consists of dopaminergic axons from the SNc and VTA that project to subcortical and cortical sites. A stainless-steel auxiliary and Ag/AgCl reference electrode combination was placed on the surface of contralateral cortical tissue -2.0 mm from bregma, and a carbon fiber

recording electrode was positioned in either the left striatum (AP +1.5, ML +0.8, and DV -2.8), NAc (AP +1.5, ML +0.8, and DV -3.8), amygdala (AP +2.4, ML +0.35, and DV -1.2), or mPFC (AP +2.35, ML +1.0, and DV -2.0) (n = 14 mice per recording site). Given our success recording stimulation-evoked dopamine in the striatal regions (Lester, Miller, Pate, & Blaha, 2008; Lester, Rogers, & Blaha, 2010), prior to recording in the amygdala or mPFC, an optimal stimulation-evoked response was found in the NAc to ensure proper placement of the stimulating electrode in the MFB. Recording electrodes were then moved to either the amygdala or mPFC. Fixed potential amperometry, also known as continuous amperometry, coupled with carbon fiber recording microelectrodes has previously been confirmed as a valid technique for real-time monitoring of stimulation-evoked dopamine release in the nucleus accumbens, striatum and other brain systems (Dugast, Suaud-Chagney, & Gonan, 1994; Forster & Blaha, 2003; Lester, Rogers, & Blaha, 2010; Suaud□Chagny et al., 1995). All amperometric recordings were made within a Faraday cage to increase signal to noise ratio. A fixed potential (+0.8 V) was applied to the recording electrode, and oxidation current was monitored continuously (10K samples/sec) with an electrometer (ED401 e-corder 401 and EA162 Picostat, eDAQ Inc., Colorado Springs, CO) filtered at 50 Hz.

Electrical stimulation and drug administration

Following surgical set-up, a series of cathodal current pulses was delivered to the stimulating electrode via an optical isolator and programmable pulse generator (Iso-Flex/Master-8; AMPI, Jerusalem, Israel). Stimulation parameters varied depending on the aspect of dopamine transmission being measured. Initially, the stimulation protocol consisted of 20 monophasic 0.5 ms duration pulses (800 μAmps) at 50 Hz every 30

seconds to establish a baseline dopamine response. To account for transient electrical stimulation artifacts, the fixed-potential of the recording electrode was also set to 0V in each brain area and the response was subtracted from recordings at +0.8 V to ensure an absolute dopaminergic baseline response is being monitored (Dugast, Suaud-Chagney, & Gonan, 1994; Benoit-Marand, Borrelli, & Gonan, 2001).

Dopamine autoreceptor sensitivity was assessed by adapting previous in vivo methods used in examination of dopaminergic neurons (Benoit-Marand, Borrelli, & Gonan, 2001). The current study applied a pair of test stimuli (T1 and T2, each 10 pulses at 50 Hz with 10 sec between T1 and T2) to the MFB every 30 sec (Fielding et al., 2013; Mittleman et al., 2011). Five sets of conditioning pulses (1, 5, 10, 20, and 40; 0.5 ms pulse duration at 15 Hz) were delivered prior to T2 such that there was 0.3 s between the end of the conditioning pulse train and initiation of T2. Autoreceptor-mediated inhibition of evoked dopamine efflux was expressed in terms of the change in the amplitude of T2 with respect to T1 for each set of conditioning pulses; low-to-high dopamine autoreceptor sensitivity was represented as low-to-high percent inhibition of evoked dopamine efflux (i.e. high sensitivity results in a lower amplitude of T2 relative to T1).

Upon completion of the autoreceptor sensitivity test, stimulation parameters were reset to 20 pulses at 50 Hz every 30 sec. Baseline levels of MFB stimulation-evoked dopamine were monitored for 10 min in each mouse prior to drug administration. From these baseline recordings, we quantified dopamine release (the magnitude of the response) and dopamine uptake, an indication of DAT functioning (measured by dopamine half-life decay, i.e. the time for 50% decrease from the maximum evoked increase to the prestimulus baseline level) (Benoit-Marand, Jaber, & Gonon, 2000;

Mittleman et al., 2011). Following this baseline recording, animals were injected systemically (i.p.) with uptake blockers. In order to validate that dopamine was the oxidized molecule being monitored in these experiments, separate groups of mice received i.p. injections of either saline (control, 0.9%), the dopamine uptake blocker nomifensine (10 mg/kg), or the selective-serotonin uptake blocker fluoxetine (10 mg/kg) and the norepinephrine uptake blocker desipramine (10 mg/kg) with a 30 min recording period following each injection (Mittleman et al., 2011; Tye, Miller, & Blaha, 2013). To reduce the number of mice used, the latter group of mice received a fluoxetine injection, followed by 30 min of amperometric recordings, then a desipramine injection, following by another 30 min recording period. Next, a 3 min continuous stimulation was applied to assess overall dopamine supply levels (Fielding et al., 2013). At conclusion of the amperometric recordings, recording electrodes were removed from the brain for in vitro calibration using dopamine solutions (0.2-1.2 μM) administered with a flow injection system (Michael & Wightman, 1999; Prater, Swamy, Beane, & Lester, 2018). Thus, change in dopamine oxidation current (μAmp) was converted to dopamine concentration (μM).

Data analysis

To quantify MFB stimulation-evoked dopamine efflux, pre-stimulation current values were normalized to zero current values and data points occurring within a range of 0.25 sec pre- and 5 sec post-onset of the stimulation were extracted from the continuous record at the desired time points. Changes in stimulation-evoked dopamine release and half-life following the drug challenge (either nomifensine, fluoxetine, or desipramine) were expressed as mean percent change relative to the pre-drug baseline response

(100%). Dependent measures used to quantify aspects of dopamine transmission included: baseline (pre-drug) dopamine release, dopamine half-life, dopamine autoreceptor-mediated inhibition, overall dopamine supply, and changes in dopamine release and half-life following uptake blockade (via systemic nomifensine, fluoxetine, or desipramine). An analysis of variance (ANOVA) was used to compare the dependent measures of dopamine transmission between brain areas (striatum, NAc, amygdala, and mPFC). Tukey's HSD post hoc tests were used to examine significant group differences indicated by $p < .05$.

Histology

At conclusion of amperometric recordings, an iron deposit was formed in brain sites by passing a direct anodic current (100 μAmps for 10 sec) through the stimulating electrode, and mice were euthanized with a 0.25 ml intracardial injection of urethane (0.345 g/ml). Brains were removed, immersed in 10% buffered formalin containing 0.1% potassium ferricyanide (which causes a redox reaction at the stimulation site resulting in a Prussian blue spot), and then stored in 30% sucrose/10% formalin solution for at least 1 week prior to sectioning. Using a cryostat at -20°C, 30 μm coronal sections were sliced, and electrode placements were determined under a light microscope and recorded on representative coronal diagrams (Paxinos & Franklin, 2001).

Drugs

Urethane (U2500), nomifensine (N1530), fluoxetine (F132), desipramine (D3900), and dopamine (H8502) were obtained from Sigma-Aldrich Chemical (St Louis, MO). All chemicals, except for urethane (distilled water) and dopamine (PBS at pH 7.4), were dissolved in saline (0.9%).

Results

Stereotaxic placements of electrodes

The tips of the stimulating electrodes were positioned within the anatomical boundaries of the MFB. The placements of the electrochemical recording electrode surfaces were confined to the core of the striatum, NAc, basolateral amygdala (BLA), or mPFC. Figure 1A-D is a depiction of the placement ranges and coordinates from bregma (Paxinos & Franklin, 2001).

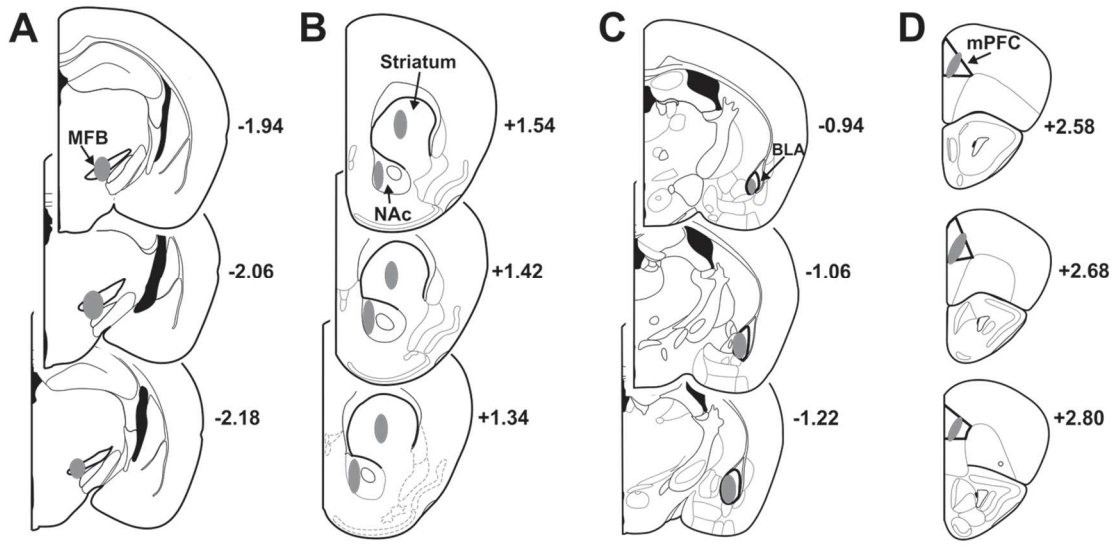

Figure 1. Representative coronal sections of the mouse brain (adapted from the atlas of Paxinos and Franklin, 2001), with black shaded areas indicating the placements of the (A) stimulating electrodes in the medial forebrain bundle (MFB) and amperometric recording electrodes in the (B) striatum, (B) nucleus accumbens (NAc), (C) amygdala, or (D) medial prefrontal cortex (mPFC). Numbers correspond to mm from bregma.

Dopamine release

Significant differences in relation to the magnitude of phasic dopamine release were observed between recording sites ($F_{(3,52)} = 12.48$, $p < 0.01$, $\eta_p^2 = 0.42$). For these analyses, baseline (pre-drug) responses were used (n = 14 per recording site). Post hoc

tests revealed that the striatum (*M* ± *SEM*: 1.87 µM ± 0.46) was statistically similar to the NAc (1.93 µM ± 0.37, $p = 0.99$), but both the striatum and NAc had significantly greater dopamine release than the amygdala (0.12 µM ± 0.02, $p < 0.01$ for both comparisons) and mPFC (0.05 µM ± 0.02, $p < 0.01$ for both comparisons) (see Figure 2). No differences in dopamine release were observed between the amygdala and mPFC ($p = 0.99$).

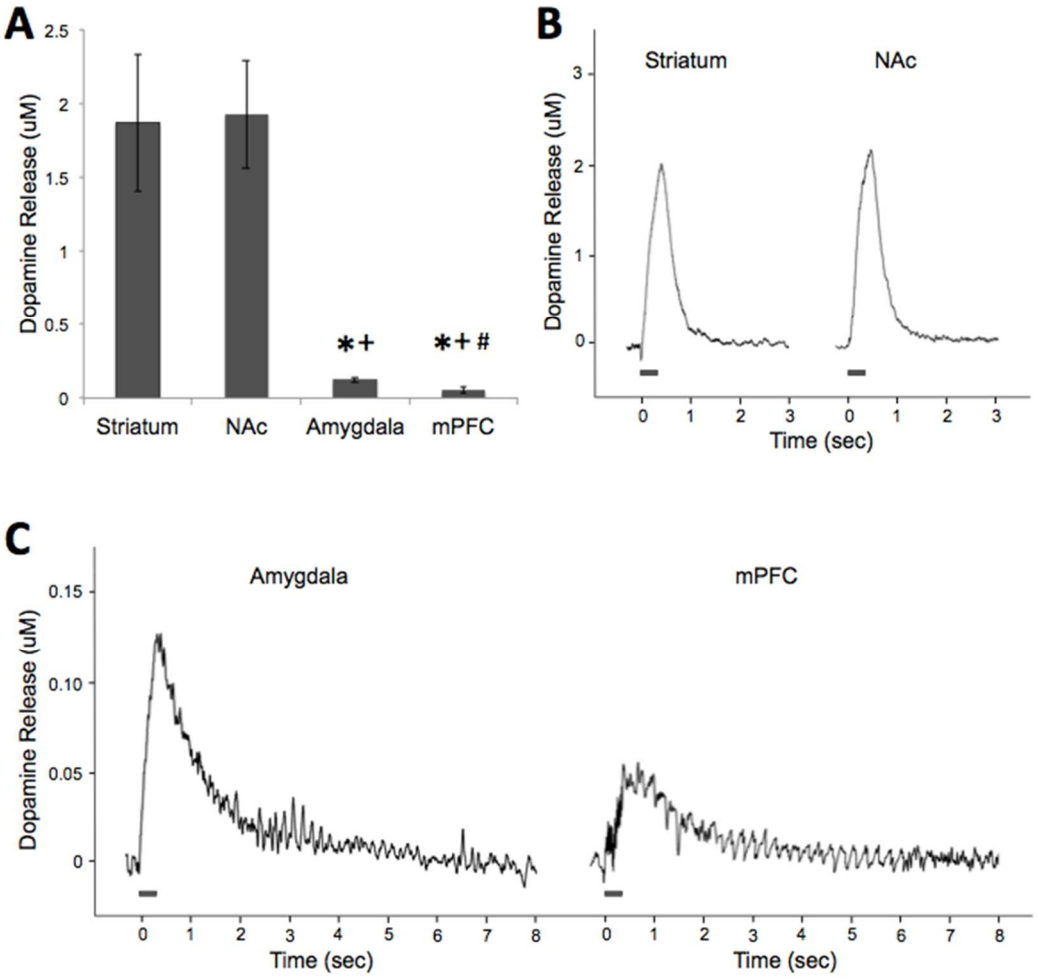

Figure 2. Amperometric recordings of stimulation-evoked dopamine release in the striatum, nucleus accumbens (NAc), amygdala, or medial prefrontal cortex (mPFC). (A) Mean (± SEM) differences were observed between brain regions. Symbols illustrate significant differences from striatum (*), NAc (+), and amygdala (#). (B and C) Profiles illustrate example responses from each recording site. Time zero indicates the start of the train of 20 pulses at 50 Hz.

Dopamine uptake

Calculating the baseline (pre-drug) dopamine half-life, i.e. the time needed for 50% decrease from the maximum evoked concentration increase to the pre-stimulus level, allowed for comparisons of dopamine uptake rates, an indication of DAT functioning, between brain areas. Significant differences in dopamine half-lives were observed between recording sites ($F_{(3,52)} = 55.67$, $p < 0.01$, $\eta_p^2 = 0.76$) (n = 14 per recording site). Post hoc tests revealed that the half-life of dopamine release in the striatum (*M* ± *SEM*: 0.40 sec ± 0.04) was statistically similar to that of the NAc (0.43 sec ± 0.05, $p = 0.99$), but both the striatum and NAc had significantly faster dopamine half-lives than the amygdala (0.86 sec ± 0.12, $p = 0.02$ and 0.04, respectively) and mPFC (2.12 sec ± 0.17, $p < 0.01$ for both comparisons). The amygdala also had a significantly faster dopamine half-life than the mPFC ($p < 0.01$) (see Figure 3A). Reduced, or smaller, dopamine half-lives indicate more effective DAT functioning.

Available dopamine supply

A 3-min continuous stimulation completely depleted dopamine release at each site. Quantification of dopamine release during this depletion provides a measure of the available dopamine neuronal reserve (Fielding et al., 2013). Significant differences in dopamine supply were observed between recording sites ($F_{(3,15)} = 5.54$, $p = 0.01$) (n = 4-5 per recording site). The striatum (*M* ± *SEM:* 11070 μM ± 1373) displayed dopamine supply levels statistically similar to the NAc (8284 μM ± 1249, $p = 0.49$) but significantly greater than that of the amygdala (5096 ± 1628 μM, $p = 0.03$) and mPFC (4004 μM ± NAc and amygdala or mPFC ($p = 0.33$ and 0.13, respectively) or between the amygdala and mPFC ($p = 0.93$).

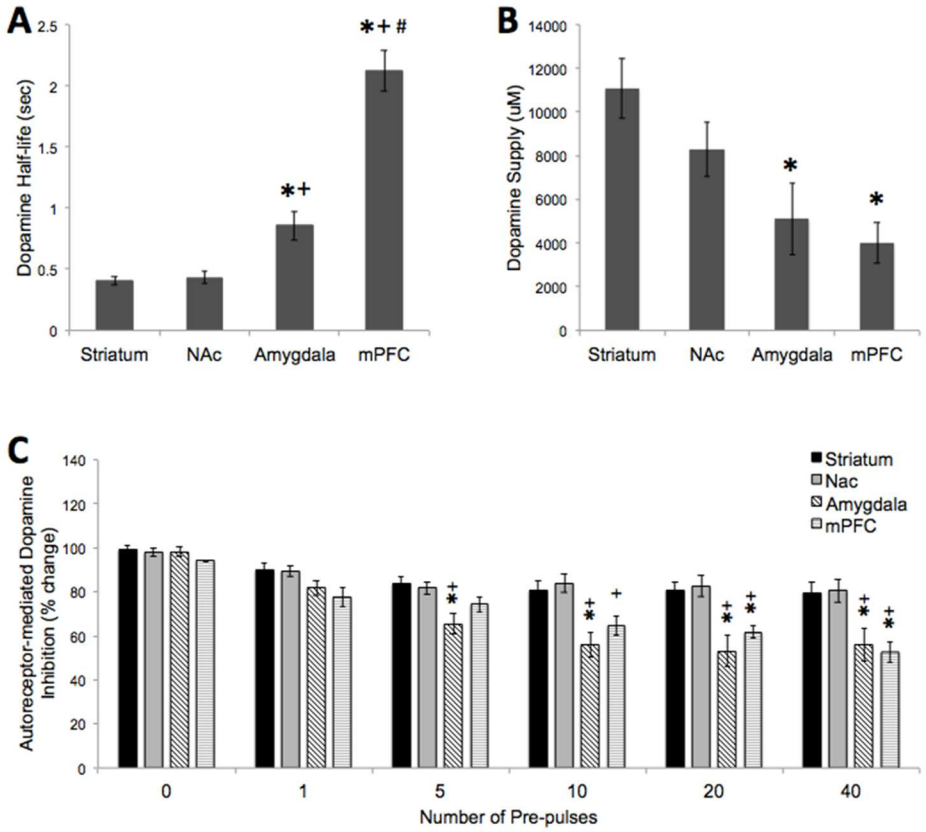

Figure 3. Specific aspects of dopamine transmission in the striatum, nucleus accumbens (NAc), amygdala, or medial prefrontal cortex (mPFC). Mean (± SEM) differences in (A) dopamine half-life, i.e. the time required for 50% decrease from the maximum evoked release to the prestimulus baseline level, (B) available dopamine supply, and (C) autoreceptor sensitivity were observed between brain regions. Symbols illustrate significant differences from striatum (*), NAc ($^{+}$), and amygdala ($^{\#}$).

924, $p = 0.01$) (see Figure 3B). No significant differences were observed between the

Dopamine autoreceptor functioning

Autoreceptor-mediated inhibition of evoked dopamine release was expressed in terms of the percentage change between test stimulations for each set of conditioning pulses or pre-pulses (n = 6 per recording site). Greater % change of the second test stimulation relative to the first test stimulation indicates more efficient or more sensitive autoreceptors. As the number of pre-pulses increases, the amount of dopamine released by those pulses also increases, leading to more autoreceptor-mediated inhibition of

subsequent dopamine release. With 0 pre-pulses, the test stimulations were separated by a sufficient amount of time that no differences were seen between the test stimulations ($F_{(3,20)} = 0.40$, $p = 0.75$, $\eta_p^2 = 0.07$); indicating the autoreceptors were not activated. Following 1 pre-pulse, the ANOVA indicated significant differences in autoreceptor inhibition levels ($F_{(3,20)} = 3.29$, $p = 0.04$, $\eta_p^2 = 0.39$); however, Tukey's HSD post hoc tests, which are known for being conservative in an attempt to control for the overall alpha level, revealed no significant differences between specific recording sites.

Dopamine autoreceptors in the striatum and NAc functioned at a statistically similar level following all pre-pulse levels (with the p value for ANOVA and post hoc analyses set at 0.05). Figure 3C exhibits M ± SEM for each group. At 5 pre-pulses, the autoreceptors in the amygdala were more active, leading to greater dopamine inhibition compared to those in the striatum and NAc, and this pattern continued through the remaining autoreceptor tests (10, 20, and 40 pre-pulses). At 10 pre-pulses, autoreceptor-mediated inhibition was greater in the mPFC compared to the striatum, and at 20 and 40 pre-pulses autoreceptor-mediated inhibition was greater in the mPFC compared to both the striatum and NAc. Dopamine autoreceptors in the amygala and mPFC functioned at a statistically similar level following all pre-pulse levels.

Pharmacological uptake blockade

During amperometric recordings of dopamine transmission, mice were given a drug challenge of either the serotonin uptake blocker fluoxetine (n = 4 per recording site), or the norepinephrine uptake blocker desipramine (n = 4 per recording site), the dopamine uptake blocker nomifensine (n = 6 per recording site), or saline (vehicle) (n = 4 per recording site). Changes in peak release and dopamine half-life 20 min post injection

were converted into percent change with baseline (pre-drug) responses being 100% (see Figure 4).

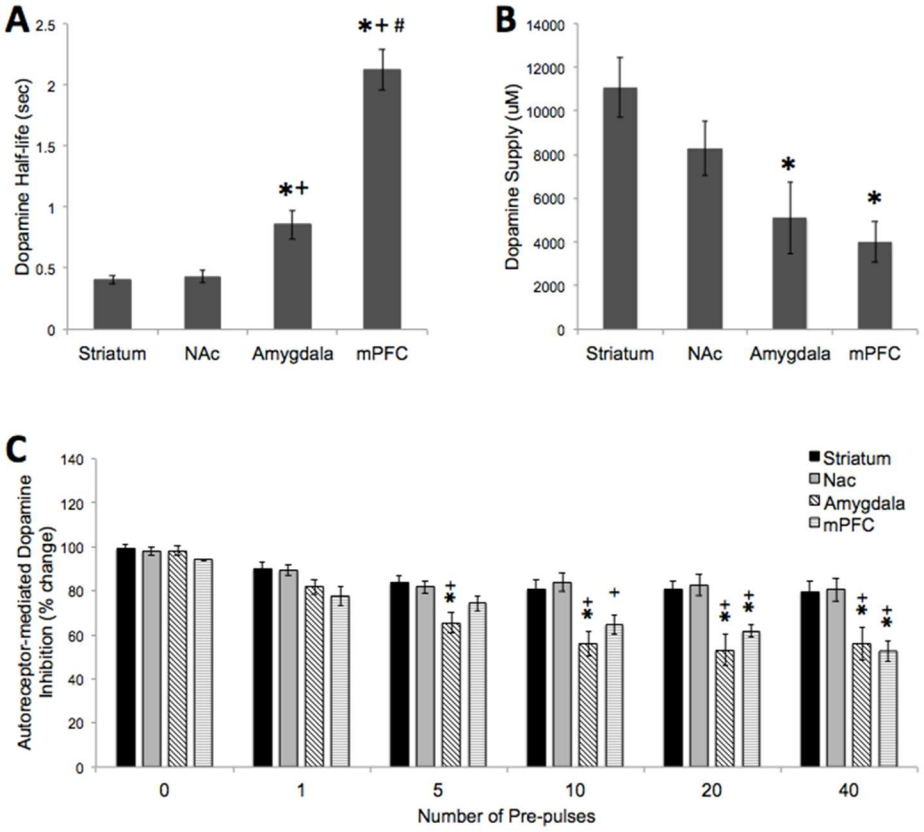

Figure 4. Amperometric recordings of stimulation-evoked dopamine release in the striatum, nucleus accumbens (NAc), amygdala, or medial prefrontal cortex (mPFC) 20 min following an intraperitoneal drug challenge of either saline (control), fluoxetine (serotonin reuptake blocker), desipramine (norepinephrine reuptake blocker), or nomifensine (dopamine reuptake blocker). (A) Nomifensine significantly increased mean (± SEM) percent changes in dopamine release relative to saline in the striatum and NAc. (B) Nomifensine significantly increased mean (± SEM) percent changes in dopamine half-life relative to saline in all brain sites. * indicates significant difference from saline.

In the striatum, systemic administration of these uptake blockers significantly altered percent change in dopamine release ($F_{(3,14)} = 7.29$, $p < 0.01$, $\eta_p^2 = 0.61$) and dopamine half-life ($F_{(3,14)} = 14.84$, $p < 0.01$, $\eta_p^2 = 0.76$). Specifically, nomifensine ($M \pm SEM$: 248% ± 45) significantly increased dopamine release in the striatum relative to

saline (96% ± 3, $p = 0.02$). Percent change in release following fluoxetine (93% ± 5) and desipramine (88% ± 4) were statistically similar to saline ($p = 1.00$ and 0.99, respectively). Regarding drug effects on dopamine half-lives in the striatum, nomifensine (377% ± 56) significantly increased dopamine's time in the synapse compared to the effect of saline (101% ± 6, $p < 0.01$), while administration of fluoxetine (102% ± 4) and desipramine (101% ± 1) again made no difference relative to saline ($p = 1.00$ for both comparisons).

In the NAc, similarly to that of the striatum, differences in percent change in dopamine release ($F_{(3,14)} = 16.56$, $p < 0.01$, $\eta_p^2 = 0.78$) and dopamine half-life ($F_{(3,14)} = 34.48$, $p < 0.01$, $\eta_p^2 = 0.88$) were observed following the systemic drug challenge. Specifically, nomifensine (329% ± 45) significantly increased dopamine release in the NAc relative to saline (91% ± 8, $p < 0.01$), but release changes following fluoxetine (94% ± 5) and desipramine (85% ± 3) were statistically similar to saline ($p = 1.00$ for both comparisons). Regarding drug effects on dopamine half-lives in the NAc, nomifensine (450% ± 46) significantly increased dopamine's time in the synapse compared to the effect of saline (102% ± 4, $p < 0.01$), while administration of fluoxetine (100% ± 6) and desipramine (101% ± 2) again made no difference relative to saline ($p = 1.00$ for both comparisons).

In the amygdala, systemic administration of these uptake blockers had no significant effect on percent change in dopamine release ($F_{(3,14)} = 0.27$, $p = 0.85$, $\eta_p^2 = 0.05$) but did significantly affect dopamine half-lives ($F_{(3,14)} = 52.89$, $p < 0.01$, $\eta_p^2 = 0.92$). Nomifensine (235% ± 12) significantly increased the dopamine half-life in the amygdala compared to saline (104% ± 6, $p < 0.01$), while neither fluoxetine (106% ± 6)

nor desipramine (103% ± 6) altered uptake kinetics relative to the saline control ($p = 1.00$ for both comparisons).

In the mPFC, similar to findings in the amygdala, no differences in percent change in release were observed following the drug challenge ($F_{(3,13)} = 0.74$, $p = 0.55$, $\eta_p^2 = 0.15$), but differences in percent change in dopamine half-lives were found ($F_{(3,13)} = 3.67$, $p = 0.04$, $\eta_p^2 = 0.46$). The increase in dopamine half-life seen following nomifensine (184% ± 29) approached statistical significance compared to the effect of saline (102% ± 16, $p = 0.053$), while administration of fluoxetine (107% ± 6) and desipramine (129% ± 16) clearly made no difference in the uptake of dopamine relative to saline ($p = 0.99$ and 0.79, respectively).

In all recording sites, systemic administration of the dopamine uptake blocker nomifensine altered either percent change in dopamine release and/or dopamine half-life relative to saline administration, while neither the serotonin uptake blocker fluoxetine nor the norepinephrine uptake blocker desipramine altered stimulation-evoked responses. The results of these pharmacological manipulations indicate the measured oxidation current changes during amperometric recordings correspond to dopamine efflux (Mittleman et al., 2011; Tye et al., 2013).

Importantly, the recording sites responded differently to nomifensine regarding the degree to which dopamine release ($F_{(3,20)} = 11.01$, $p < 0.01$, $\eta_p^2 = 0.62$) and dopamine half-life ($F_{(3,20)} = 10.68$, $p < 0.01$, $\eta_p^2 = 0.62$) were affected. Regarding percent change in dopamine release following nomifensine, post hoc tests revealed that the striatum responded statistically similarly to the NAc ($p = 0.37$), while nomifensine increased dopamine release more significantly in both the striatum and NAc relative to the

amygdala ($p = 0.04$ and 0.01, respectively) and mPFC ($p = 0.02$ and 0.01, respectively). No differences in percent change in dopamine release were observed between the amygdala and mPFC following nomifensine ($p = 0.99$). Regarding percent change in dopamine half-life, post hoc tests revealed that the striatum responded statistically similarly to the NAc ($p = 0.60$) and amygdala ($p = 0.09$) but to a greater degree than the mPFC ($p < 0.01$). The NAc, however, responded significantly greater, meaning nomifensine made a larger impact on dopamine half-life, compared to the amygdala and mPFC ($p < 0.01$ for both comparisons). No differences were found in percent change in dopamine half-life following nomifensine ($p = 0.54$) between the amygdala and mPFC.

Discussion

Many lines of evidence support that midbrain dopamine neurons do not subserve a single function, but drive multiple functions including motor movements, reward, attention, anxiety, and decision-making. These differing behavioral processes emerge from heterogeneous variations in uptake and release sites at midbrain dopamine afferents, which lead to site-specific differences in dopamine concentrations during synchronous phasic firing (Venton et al., 2003). Altered profiles of dopamine transmission can be seen in disorders such as Parkinson's disease, addiction, ADHD, and schizophrenia (Davis & Khan, 1991; Dougherty et al., 1999; Kish, Shannak, & Hornykiewicz, 1988). The current paper systematically examined specific aspects of dopamine transmission in 4 brain regions, the dorsal striatum, NAc, amygdala, and mPFC in healthy mice. All of these sites are known to be regulated to a large degree by dopamine and have shown pathologies in above-mentioned disorders. Dopamine operates at the synapse on the order of milliseconds, and the magnitude and timing of phasic release is critical for

behavioral conditioning (Tsai et al., 2009). Thus, in vivo fixed potential amperometry is a highly suitable technique for these measurements due to its high temporal resolution. Results from the current study suggest distinct similarities in phasic signaling between the striatum and NAc, and separately the amygdala and mPFC.

Dopamine release

Dopamine release was quantified as a function of peak height following electrical stimulation of the MFB. The present findings show that even with a consistent electrical stimulation of the dopamine axons themselves, the magnitude of dopamine release is not equivalent across all projected brain regions. The striatum and NAc share similar dopamine release concentrations, which are roughly 10-fold and 37-fold higher than that of the amygdala and mPFC, respectively. Similarly proportioned differences in phasic dopamine release have been found between these brain regions using fast scan cyclic voltammetry (Garris & Wightman, 1994). These findings are not surprising as the striatum and NAc are more densely innervated by dopamine axons than the amygdala and mPFC (Doucet, Descarries, & Garcia, 1986; Descarries, Lemay, Doucet, & Berger, 1987). Extracellular neurotransmitter concentrations from synaptic release are regulated by the rate of dopamine uptake, capacity of neuronal reserves, and autoreceptor functioning (Nicholson, 1995; Pothos, Davila, & Sulzer, 1998; Roth, 1984); these factors also likely contribute to the observed dopamine release differences in these brain sites.

Dopamine uptake

Quantification of stimulation-evoked dopamine half-life, i.e. the time for 50% decrease from the response peak to the pre-stimulus baseline level, allowed for comparisons of dopamine uptake rates, an indication of DAT functioning, in each

examined brain area. DATs help to regulate the spatiotemporal activity of dopamine in the synaptic space by swiftly returning the molecule to the presynaptic terminal and are common targets of drugs of abuse such as cocaine and amphetamine (Giros et al, 1996). Continuous amperometry has previously been used to examine dopamine activity in mice lacking the dopamine transporter, finding the half-life to be two orders of magnitude greater and diffusion distance ten times higher than in wildtypes (Benoit-Marand, Jaber, & Gonon, 2000). The current experiment found the striatum and NAc to have similarly fast dopamine half-lives, while those in the amygdala and mPFC were 2 and 5 folds longer, respectively. These clearance rates correspond to high densities of DAT in the striatum and NAc compared to the amygdala and mPFC (Aggarwal & Wickens, 2011; Sesack, Hawrylak, Matus, Guido, & Levey, 1998). Similar uptake profiles have been found using FSCV, leading to the conclusion that dopamine transmission in the striatum and NAc are more uptake-dominated while that of the amygdala and mPFC are more release-dominated (Garris & Wightman, 1994; Rice & Cragg, 2008; Stamford, Kruk, Palij, & Millar, 1988). These findings suggest that dopamine transmission in the amygdala and mPFC has the potential to diffuse further distances than that of the striatum and NAc. The median dopamine diffusion distance can be estimated from the equation $(Dt_{1/2})^{1/2}$ (Garris & Wightman, 1994; Lu, Peters, & Michael, 1998), where D is the diffusion coefficient of dopamine in the extracellular space and $t_{1/2}$ is the measured half-life of stimulation-evoked dopamine. Using the value 2.4 x 10^{-6} cm^2/s for D (Nicholson & Rice, 1991) and quantified dopamine half-lives from the present study (see Figure 3A), the calculated mean diffusion distance of dopamine molecules in each brain area is as follows: 9.8 um in the striatum, 10.1 um in the NAc, 14.4 um in the amygdala, and 22.56

um in the mPFC. These numbers are interesting for comparisons between brain sites but may not reflect absolute diffusion distances given that the diffusion coefficient used above was quantified from dopamine in extracellular striatal regions (Nicholson & Rice, 1991). Tortuosity, a measure of the extent to which diffusing particles are hindered by the presence of obstructions (in the form of cells or their extensions), is a major component of the equation defining the diffusion coefficient and may differ between these brain sites (Nicholson & Phillips, 1981; Pereira, Oliveira, & Rosado, 2006).

DATs are typically depicted as clearance mechanisms for dopamine at the synapse to restrict the amount of spillover and active synaptic lifetime of dopamine, thus serving as a limiting factor for point-to-point synaptic contacts (Ciliax et al., 1995). A more accurate depiction of the role of DATs is emerging, highlighting their critical role in influencing the 3-dimensional sphere of diffusion beyond the synaptic release site. Reduced DAT functioning leads to greater diffusion distances and a greater sphere of influence through volume transmission. In contrast to point-to-point synaptic contacts, volume transmission provides a communication mode that is temporally slower, broader in anatomical reach, and more suited to modulatory/tuning functions. Sesack and colleagues (1998) suggested that the elongated extracellular diffusion in the prelimbic PFC results from a shortage of DATs in mesorcortical dopamine axons, as well as DATs distributed further from the synaptic release site. Our results also support computational models which state that slow reverberatory dynamics, operating on a timescale up to seconds, are characteristic of mPFC microcircuits that underly working memory and decision-making computations, with dopamine serving a well-recognized role in these processes (Shephard & Grillner, 2018; Wang, 2006). On the contrary, the striatal areas

have more restricted axonal distributions of DATs, which may account for a more confined diffusive outreach to postsynaptic receptors (Cragg & Rice, 2004). The results of the present study indicate that dopamine uptake rates in the amygdala fall between those of the striatal regions and mPFC. Extending interpretations of DAT properties and diffusion distances to dopamine transmission in the amygdala suggests brain alterations related to fear, anxiety, memory storage, and other emotional processing may be longer lasting and potentially more impactful than the rapid, movement and reward-related tasks performed by the striatum and nucleus accumbens (for review see Schultz et al., 2007).

Dopamine supply

In vivo research shows the extracellular dopamine concentration levels are clearly increased when DAT blockers or other dopamine agonists are administered (Lester, Rogers, & Blaha, 2010; Robinson & Camp, 1990). This suggests that each dopamine terminal has a reserve of transmitter available for release when challenged environmentally or experimentally, via pharmaceuticals or electrical stimulation (Pothos, Davila, & Sulzer, 1998; Willuhn, Burgeno, Groblewski, & Phillips, 2014). The differing degrees of dopamine release seen in the striatum, NAc, amygdala, and mPFC, may be due, in part, to different capacities of neuronal dopamine reserves in these regions. The present study incorporated an established means of quantifying available dopamine supply *in vivo* (Fielding et al., 2013). A continuous stimulation (50 Hz) lasting 3 min was applied to the MFB during dopamine recordings in each brain site. The striatum and NAc both have a similar, substantial reserve of dopamine available for release, while the amygdala and mPFC have also have similar levels of available dopamine supply but roughly half that of the striatal areas. Again, this finding likely relates to the number of

dopamine axons innervating these brain regions (Doucet, Descarries, & Garcia, 1986; Descarries, Lemay, Doucet, & Berger, 1987). A relatively small reserve of available dopamine may have driven these systems to use the neurotransmitter more efficiently, for instance lower release concentrations with longer dopamine half-lives and increased diffusion distances, as a way of preserving energy and resources (Attwell & Gibb, 2005). Of course, this phenomenon may be flipped or even bidirectional, meaning reduced release concentrations and longer half-lives may also influence dopamine synthesis and supply levels. Either way, maximum stimulations of the MFB have varying effects on these brain sites.

Dopamine autoreceptor functioning

Presynaptic inhibition allows for neurons to modulate their own neurotransmitter release through the function of neurotransmitter-specific autoreceptors, which in vivo have been shown to be activated by stimulation-evoked dopamine overflow (Dugast et al, 1997; Stark et al., 1989). In vivo, the onset of D2 inhibition is maximal between 150 and 300 ms after the end of the initial conditioning stimulation (Benoit-Marand, Borrelli, & Gonan, 2001). D2 receptors are found in high density in the striatum and NAc, and to a lower extent in the amygdala and mPFC (Bouthenet et al., 1991; Ford, 2014; Meador-Woodruff et al., 1989). The current study examined autoreceptor sensitivity in the striatum, NAc, amygdala, and mPFC by using five sets of conditioning pulses or pre-pulses (1, 5, 10, 20, and 40 pp), delivered 300ms prior to the second test stimulation (T2). T2 was then compared to an identical prior stimulation (T1) which did not follow pre-pulses. Low-to-high autoreceptor sensitivity is indicated by low-to-high percent

inhibition of evoked dopamine efflux, such that high sensitivity would result in a lower amplitude of T2 relative to T1.

During the 0 or 1 pre-pulse train, all sites exhibited the same level of dopamine inhibition, which was neglible at that point; however, as the number of pre-pulses increased the amygdala and mPFC (starting at 5 and 10 pre-pulses, respectively) began to exhibit greater levels of inhibition or higher autoreceptor sensitivity when compared to the striatum and NAc. This pattern remained statistically significant throughout the test. The greatest amount of autoreceptor-mediated inhibition was seen at 40 pre-pulses, with the inhibition level at the amygdala and mPFC being nearly double that of the striatum and NAc. Given that the present study also showed that the amygdala and mPFC have nearly half the dopamine supply compared to the striatal areas, more sensitive autoreceptors may be another frugal mechanism to preserve energy and resources. Autoreceptors in the amygdala and mPFC may also help account for the reduced DAT functioning in these regions. Studies have shown that following chronic dopamine agonism, the D2 autoreceptors in DAT knockout mice become supersensitive (Jones et al., 1999; King et al., 1999). More intensive autoinhibition may be a hallmark of systems with less efficient uptake, since these types of neural schemes tend to entail a greater sphere of influence and longer duration of dopamine in the synaptic space.

Dopamine uptake blockade

Many drugs, both illicit (such as cocaine and methamphetamine) and prescribed (such as Ritalin and Adderall), act by blocking dopamine uptake, thereby increasing and prolonging the effect of dopamine in the extracellular space. Dopamine transmission in the striatum and NAc are the most highly studied brain regions for these particular drugs

due to the known influence of these brain areas on reward, addiction, and attention (Church, Justice, & Byrd, 1987; Schultz, 1998; Volkow et al., 1997). Indeed, results obtained in the present study showed that the dopamine uptake blocker nomifensine increased the magnitude and half-life of dopamine efflux in these regions. Findings such as these have led to the popular notion that drugs of abuse commandeer the brain's natural reward system during the addictive process (Kauer & Malenka, 2007). Accordingly, prevention of dopamine uptake also increases diffusion distances, leading to activation of far more dopamine receptors at distal sites and alteration the normal balance of tonic and phasic dopamine activity (Venton et al., 2003; Sulzer, 2011). These drugs are not spatially restricted in the brain when administered and, therefore, are potentially influential wherever DATs are located.

The present study found that the dopamine uptake blocker nomifensine had no effect on the magnitude of dopamine release in the amygdala or mPFC. Similar findings have been reported when using FSCV, leading researchers to conclude that DAT inhibitors may not be as influential in these brain areas (Cass & Gerhardt, 1995; Garris, & Wightman, 1995; Jones, Garris, Kilts, & Wightman, 1995; Mundorf, Joseph, Austin, Caron, & Wightman, 2001). Given that DAT inhibitors specifically target proteins regulating dopamine uptake kinetics, but not necessarily release, analysis of dopamine half-life is considered to provide a more appropriate measure. In the present study, systemic injection of the dopamine uptake blocker nomifensine nearly doubled the time required for dopamine clearance in both the amygdala and mPFC, suggesting such drugs are also influential in these brain regions although to a lesser degree than in the striatum and NAc. The vast differences between the effect of DAT inhibition on dopamine

release and half-life in striatal and corticolimbic regions is likely again related to terminal density of DAT (Garris & Whiteman, 1994), and also the relative affinities of DAT in each of these regions. Specifically, DAT in the striatum and NAc have a relatively high affinity (Km of ~0.2 uM and a Vmax of 3.8 uM/s and 2.4 uM/s, respectively), while that of the amygdala has been shown to be lower (Km of 0.6 uM and Vmax of 0.49 microM/s) (Horn, 1990; Jones, Garris, Kilts, & Wightman, 1995). As another mechanism to consider, norepinephrine transporter (NET) levels are higher in the PFC, relative to DATs, with the opposite being true in subcortical regions (Morón, Brockington, Wise, Rocha, & Hope, 2002; Sesack, Hawrylak, Matus, Guido, & Levey, 1998). Further, dopamine has been shown to have a higher affinity for the NET than the DAT (Morón, Brockington, Wise, Rocha, & Hope, 2002).

Overall, these findings and others suggest that uptake blockers have spatially heterogeneous effects in different regions of the brain. These findings provide implications for education on the use of recreational and medicinal drugs which affect DAT functioning and, consequentially, extracellular dopamine levels. For example, increased dopamine activity in the amygdala can lead to increased occurrence of anxiety-related behaviors (Abercrombie, Keefe, DiFrischia, & Zigmond, 1989; Borowski & Kokkindis, 1998; de la Mora et al., 2010), potentially by dampening the regulatory control of PFC inputs to the amygdala (Diaz et al., 2011). Furthermore, excessive dopamine in the mPFC can result in poorer performance on cognitive tasks related to working memory and attention (Mattay et al. 2003; Zahrt, Taylor, Mathew, & Arnsten, 1997). Thus, an understanding of phasic dopamine manipulations in these regions is critical for symptom control.

Conclusions

The present study found distinct differences in phasic dopamine dynamics in 4 prominent output sites for the nigrostriatal and mesolimbic dopamine pathways: the dorsal striatum, NAc, amygdala, and mPFC. Specifically, the striatum and NAc had increased stimulation-evoked phasic dopamine release, faster dopamine uptake (leading to less dopamine diffusion), weaker autoreceptor functioning, greater supply levels of available dopamine, and increased dopaminergic responses to DAT blockade compared to the amygdala and mPFC. Previous studies have measured dopamine release and uptake differences in these sites, but to our knowledge this paper may be the first to systematically quantify these properties while concurrently examining autoreceptor functioning, dopamine supply levels, and the effect of uptake blockers in mice using a consistent electrochemical technique with high temporal resolution. Our findings revealed paired similarities in phasic dopamine dynamics between the striatum and NAc and between the amygdala and mPFC. Functionally, these brain sites influence a range of different behaviors; however, anatomically, the striatum and NAc exist along a continuum with the NAc often referred to as the ventral striatum. Other researchers have likewise proposed portions of the amygdala to be a neural extension of the frontal cortex, rather than an independent functional unit (LeDoux, 2007; Swanson & Petrovich, 1998). Anatomical similarities also include the number of dopaminergic projections to these regions, with the striatum and NAc being more densely innervated by dopamine terminals than the amygdala and mPFC (Doucet, Descarries, & Garcia, 1986; Descarries, Lemay, Doucet, & Berger, 1987). Overall, these findings indicate that phasic dopamine may have different modes of communications between striatal and corticolimbic regions,

with the first being profuse in concentration, rapid, and synaptically confined, and the second being more restricted in concentration but longer lasting and spatially dispersed. Understanding the various aspects of regional differences in phasic dopamine transmission may be useful for predicting and manipulating the effects of drugs on dopamine dependent behaviors.

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CHAPTER 3: CEREBELLAR DOPAMINERGIC SIGNALING IN THE DORSOMEDIAL STRIATUM OF FRAGILE-X MICE: SIGNIFICANCE TO AUTISM SPECTRUM DISORDERS

Introduction

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. Within this network, 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. The current study 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 aberrant neural development to the pathologies 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.

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). 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 that 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, and Blaha (2008) used DN electrical stimulation

to evoke 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 was found in both mutant mice strains, 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).

Mutant mice strains show neuronal pathology similar to those seen in autism, and behavioral deficits in these mice have also been found to correlate with those seen in

autism. Atypical behavior in children with autism may manifest as alterations in eating, increased 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, neurochemical malfunction 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 had 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 is more applicable. This is discussed more fully below.

Rodent Models of Autism Spectrum Disorders

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 Lurcher mutant mice have

frequently been used to study autism, they have an autosomal dominant mutation which causes total degeneration of 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 was chosen as the animal model for the current study.

Combining Experimental Approaches

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 (Mckimm et al., 2014; Rogers et al., 2013). 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.

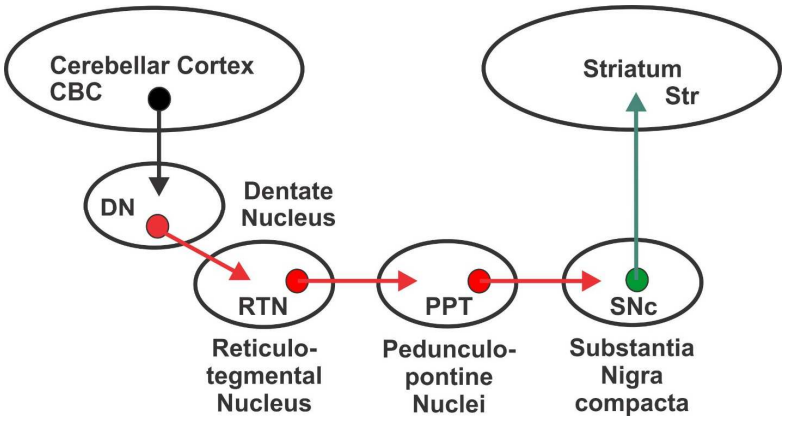

Figure 1. We predict that the cerebellum acts as a modulator of striatal dopamine release. This projected system occurs via polysynaptic inputs from cerebellar nuclei to dopamine-containing cells in the substantia nigra (SN), eventually leading to DA release in the dorsomedial striatum. Glutamatergic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

Relevance of the Striatum

Modulation of the medium spiny neurons of the dorsal striatum allows for activation and inhibition 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 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 it also has developed connections to the associative cortex and many other neuronal sectors (hippocampus, amygdala, prefrontal cortex, thalamus) (Graybiel, Aosaki, Flaherty, & Kimura, 1994). This allows for control of adaptive voluntary movement 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 regard 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 the 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 examined (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 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.

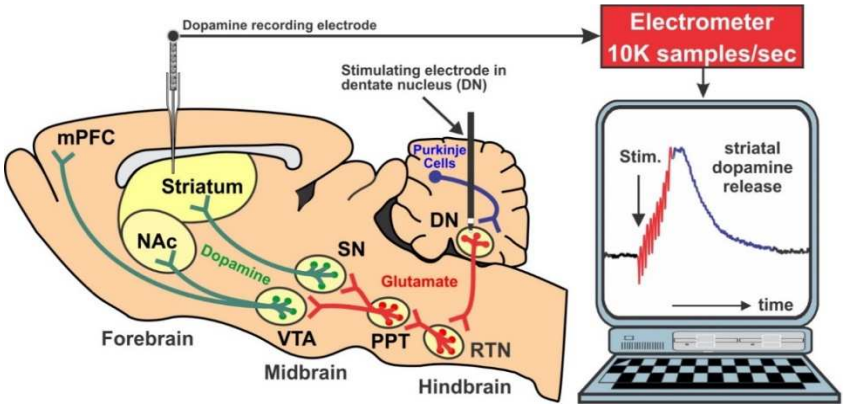

Figure 2. A stimulating electrode was placed in the deep cerebellar nuclei (dentate nucleus) and a carbon-fiber recording electrode monitored dopamine release in the dorsomedial striatum.

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 oneway 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 versus 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 ($M = 0.0021$, $SD = 0.0009$, $SEM = 0.0003$) and the FMR1 KO groups ($M = 0.0018$, $SD = 0.0027$, $SEM = 0.0011$). The two groups of mice were not found to be different with respect to the magnitude of DA release [$F(1, 13) = 4.67$, $p = 0.83$]. 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.

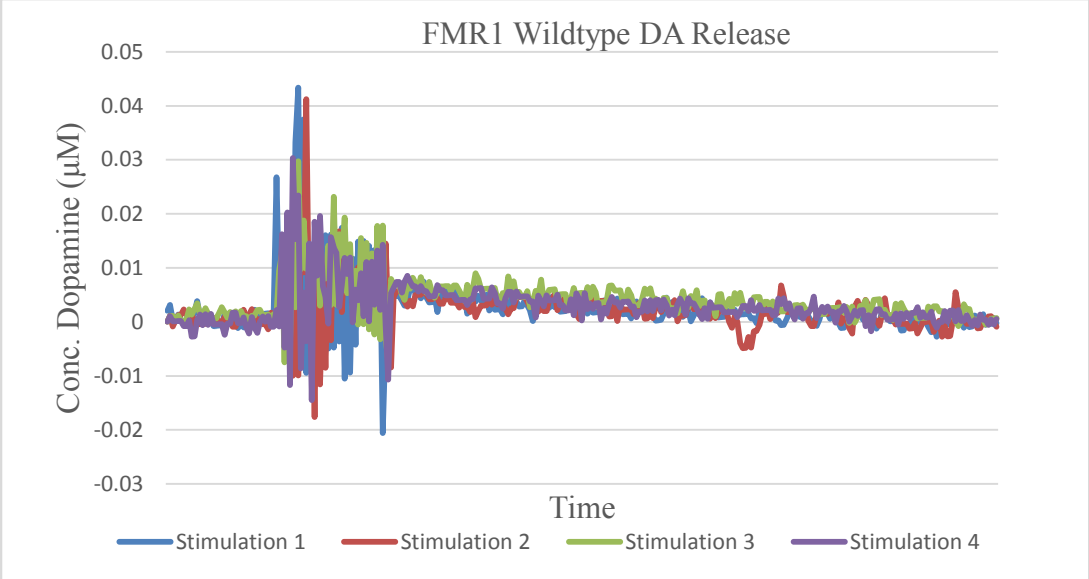

Figure 3. Recorded magnitude of DA release in FMR1 wildtype 2 seconds pre-stimulation to 30 seconds post-stimulation. Concentration of neurotransmitter is shown in micromoles (µM).

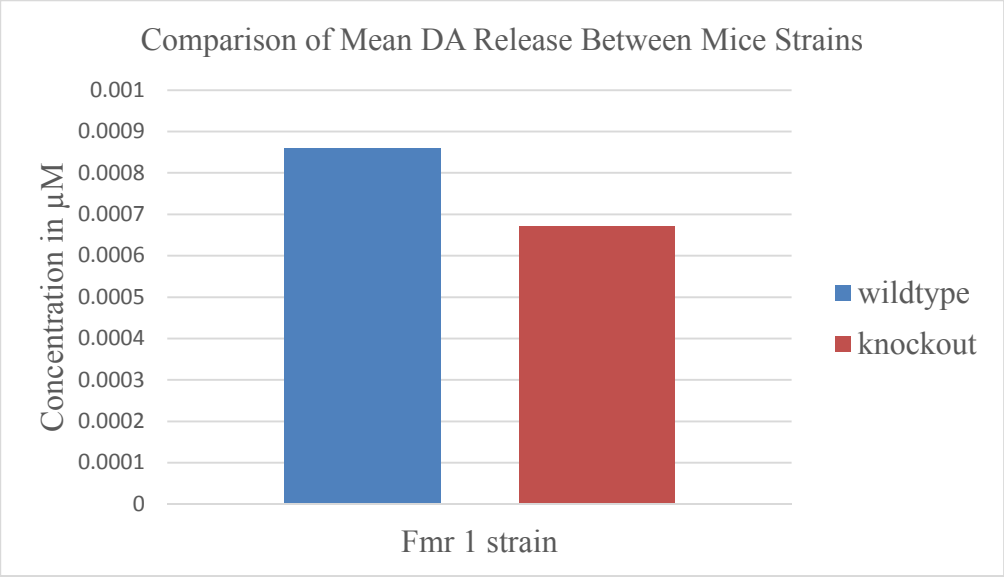

Figure 4. Average stimulation-evoked dopamine release in Fragile-X mutants (M = 0.00183 µM) and wildtypes (M = 0.002063 µM) in micromoles. No significant differences were found between groups.

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 functionally regulated by the cerebellar dentate.

It would be beneficial to examine other rodent models of autism, particularly Lurcher mutants, to determine the functionality of the dorsomedial striatum. Rogers et al. (2013) have shown that both Lurcher and FMR1 mutant mice exhibit 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) may provide further insights 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, researchers 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 next 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 worth examining.

This experiment additionally sought to obtain levels of DA release, but we were unable to assess 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 could 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, connectivity has extended to areas not seen in typically developing children. Interestingly, the striatum was found to have 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. The dorsomedial striatum, with its extensive connections throughout the brain, 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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CHAPTER 4: CEREBELLAR MODULATION OF MESOLIMBIC DOPAMINE TRANSMISSION IS FUNCTIONALLY ASYMMETRICAL

Introduction

Cerebellar and Cerebral Networks

The bilateral cerebral and cerebellar hemispheres are asymmetrical in structure, behavior, and function (Hu, Shen, & Zhou, 2008; Hugdahl & Davidson, 2004; Scott et al., 2001; Toga & Thompson, 2003). No longer considered a structure primarily for motor coordination, the cerebellum is now known to contain three distinct regions that contribute to sensorimotor, limbic, and cognitive processes (Schmahmann & Caplan, 2006). Cerebellar and cerebral systems work in concert to sharpen the timing of these neural operations (Heck et al., 2013; Weaver, 2005), and each cerebellar hemisphere is connected to multiple closed-loop cortical neural networks in the contralateral cerebral hemispheres, providing an anatomical basis for a cerebellar role in cognition (Buckner, 2013; Middleton & Strick, 1994; Schmahmann, 2016). This allows for a cerebellar mirroring of functional specializations in the cerebrum, and accordingly, lateralized cerebellar lesions produce cognitive deficits similar to those observed following lesions of the contralateral cerebral cortex (Riva & Giorgi, 2000).

Functional Asymmetry in Behavior

Hemispheric specializations have long been documented within cerebrocerebellar networks in many species including birds, rodents, and primates (Camp, Robinson, & Becker, 1983; Walker, 1980). Clinical and preclinical studies support the association of the left cerebral hemisphere with communication functions and the right cerebral hemisphere with spatial reasoning (Denenberg, 1981; D'Mello & Stoodley, 2015). Due

to contralateral connections between cerebrocerebellar systems, the cerebellar hemispheres parallel these specializations. Correspondingly, imaging and lesion studies in humans have found the left cerebellar hemisphere to be involved in visuo-spatial operations (Imamizu et al., 2003; Marien, Engelborghs, Fabbro, & De Dyn, 2001; Silveri, Misciagna, Leggio, & Molinari, 1997; Stoodley & Schmahmann, 2009), and a right cerebellar involvement in language processes (De Smet, Paquier, Verhoeven, & Marien, 2013; Papthanassiou et al., 2000; Verly et al., 2014). Likely related to these behaviorally based asymmetries, the bilateral hemispheres of the brain also contain lateralized neurotransmitter systems in cortical and subcortical regions, and certain experiences have shown to enhance this lateralization. For example, rats that were handled in their early life showed a significant left/right asymmetry (R>L) in dopamine levels in the NAc (Camp, Robinson, & Becker, 1984). Other studies in rats show greater concentrations of DOPAC/DA in the right cortex and nucleus accumbens in comparison to systems in the left hemisphere (Rosen et al., 1984). Dopaminergic lateralization may therefore be contributing to behavioral abnormalities. For instance, increased dopamine levels in the right prefrontal cortex of adult rats was found to be strongly correlated with anxiety responses in the elevated plus-maze test (Andersen & Teicher, 1999). Furthermore, researchers suggest that heterogeneous profiles of dopamine are related to handedness or limb preference; dopamine levels tend to be greater in the NAc ipsilateral to the preferred limb (Budlin et al., 2008). Asymmetrical structure and function in dopaminergic systems appear to be a product of both life experiences and typical neurodevelopment.

Cerebellar Involvement in Dopamine Asymmetry

Many neurophysiological disorders are characterized by altered profiles of mesocorticolimbic dopaminergic transmission, such as addiction, ADHD, and schizophrenia (Davis & Khan, 1991; Dougherty et al., 1999; Kish, Shannak, & Hornykiewicz, 1988), and interestingly, cerebellar pathology and specifically Purkinje cell dysfunction are being considered as substrates in these and other psychiatric disorders (Shakiba, 2014; Fatemi, & Folsom, 2014; Wang, Kloth, & Badura, 2014). The cerebellum exerts modulatory influence on the cerebrum via Purkinje cells and their synaptic output on the deep cerebellar nuclei, specifically the dentate nucleus (DN), which provides the sole output from the cerebellum to the cerebrum. Mittleman, Goldowitz, Heck, and Blaha (2008) used DN electrical stimulation to evoke dopamine efflux in the medial prefrontal cortex (mPFC) of Lurcher mutant mice (a common model of autism spectrum disorder with 100% loss of Purkinje cells within the first 4 weeks of life). The Lurcher mutants exhibited an attenuation in mPFC dopamine release compared to controls, suggesting that developmental loss of Purkinje cells in the cerebellum, similar to that of autism spectrum disorder, can lead to a disruption in mPFC dopamine transmission.

Rogers et al. (2011) expanded on the research of Mittleman and colleagues by using in vivo fixed potential amperometry (FPA) with carbon-fiber microelectrodes to compare functional adaptations of cerebello-cortico circuitry associated with abnormalities in cerebellar functioning. They found attenuation and reorganization of cerebellar modulation in the mPFC dopamine release of Lurcher mutants and Fmr1 mice (another genetic model that exhibits dysfunction or absence of Purkinje cellular influence

in the cerebellum). The reorganization of these pathways, which originated in the DN and concluded in the mPFC, included altered relative influence of the VTA and thalamic nuclei, with the mutant mice showing a stronger dependence on thalamic nuclei compared to control mice (Rogers et al., 2011). This shift in cerebellar modulation towards the ventral lateral thalamus leads to speculation about the cerebellum's influence on not only the mPFC, but also the nigrostriatal and mesolimbic pathways (Di Martino et al., 2011). The current experiment adds to the literature supporting a cerebellar role in modulation of cerebral neurotransmission and provides evidence that dopamine lateralization may be modulated or organized by the cerebellum.

Intertwinement of the VTA within cerebrocerebellar networks suggests that the cerebellum is in position to modulate dopamine release not only in the mPFC but also in the NAc, the other major projection site of the mesocorticolimbic dopamine system. Neural fibers between the VTA and NAc constitute one of the most densely innervated dopamine pathways in the brain (Doucet, Descarries, & Garcia, 1986; Descarries, Lemay, Doucet, & Berger, 1987). Dopamine release in the NAc is known to be associated with reward and motivational processes (Cohen et al., 2012; Matsumoto & Hikosaka, 2009; Mirenowicz & Schultz, 1996; Robinson et al., 2009), and disruption to normal dopamine processing, including hemispheric balance, can lead to a host of motor and cognitive deficits. For example, decreased motivation and novelty seeking are related to asymmetry of dopamine often observed in patients with Parkinson's disease (Tomer & Aharon-Peretz, 2004), and individual differences in incentive motivation or sensitivity to natural rewards in humans has been associated with increased asymmetry in dopaminergic systems (Tomer et al., 2008).

Specific Aims of the Experiment

Aforementioned findings highlight the importance of the functional balance in dopamine transmission and the influence of the cerebellum on dopamine cell bodies. Overall, the present study aimed to determine whether the cerebellum output can stimulate NAc dopamine release as has been shown with mPFC and whether hemispheric asymmetry occurs between these pathways. Determining if reward processes are lateralized may have considerable application to our understanding of normal and abnormal psychological states. The present study includes three series of tests that were conducted to address three separate hypotheses. In the first experiment, we assessed subcortical dopamine lateralization by quantifying NAc dopamine release in each hemisphere elicited via stimulation of the medial forebrain bundle (MFB), which consists of the dopaminergic axons projecting from the VTA to NAc. The second experiment assessed cerebellar influence of NAc dopamine lateralization by comparing DN stimulation-evoked dopamine release in both hemispheres. The DN has contralateral glutamatergic projections to reticulotegmental nuclei (RTN) that, in turn, project to pedunculopontine nuclei (PPT), which projects to and stimulates dopamine cell bodies in the VTA. For this reason, the present study stimulated the DN located contralateral to the NAc recording site (left DN stimulation with right NAc recording and vice versa) (Bostan, Dunn, & Strick, 2010; Palesi et al., 2015). The third experiment in the present study examined the potential cross-hemispheric influence of cerebellar DN on this dopaminergic pathway. During contralateral DN stimulation-evoked dopamine recordings, separate groups of mice received an infusion of either lidocaine or phosphate-buffered saline (PBS; vehicle control) into the ipsilateral DN. These present experiments

help improve our understanding of dopamine lateralization and the relationship between the cerebrocerebellar networks, both of which may provide targets for pharmacological interventions in neuropathologies related to dopamine dysfunction.

Materials and Methods

Animals

Thirty-two male C57BL/6J mice (Jackson Laboratories, ME) were housed 3-5 per cage in a temperature-controlled environment (21±1 °C) on a 12 hr light/dark cycle with (lights on at 0600) and given food and water available ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Memphis and conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Efforts were made to reduce the number of animals used and to minimize pain and discomfort.

Surgery

Mice were anesthetized with urethane (1.5 g/kg, i.p.) and placed in a stereotaxic frame (David Kopf Instruments, Tujundga, CA) ensuring a flat skull. Body temperature was maintained at 36±0.5 °C with a temperature-regulated heating pad (TC-1000; CWE, NY). All stereotaxic coordinates are in mm from bregma, midline, and dura according to the mouse atlas of Paxinos and Franklin (2001). In each mouse, a concentric bipolar stimulating electrode (SNE-100, Rhodes Medical, CA) was implanted into either the left cerebellar DN (AP +6.25, ML +2.0, and DV -2.0), or right DN (AP +6.25, ML -2.0, and DV -2.0), or either the left MFB (AP +2.0, ML +1.1, DV -4.0) or right MFB (AP -2.0, ML +1.1, DV -4.0). A stainless-steel auxiliary and Ag/AgCl reference electrode combination was placed on the surface of cortical tissue contralateral to the stimulation

electrode and -2.0 mm from bregma, and a carbon fiber recording electrode was positioned in either the left NAc (AP +1.5, ML +1.0, and DV -4.0) or the right NAc (AP +1.5, ML -1.0, and DV -3.8). For MFB stimulations, the recording electrode was placed in the ipsilateral NAc; however, due to contralateral connections in cerebrocerebellar circuity, the recording electrode was placed contralateral to cerebellar DN stimulation (Bostan, Dunn, & Strick, 2010; Palesi et al., 2015).

Fixed-potential Amperometry

Fixed potential amperometry coupled with carbon fiber recording microelectrodes has been confirmed as a valid technique for real-time monitoring of stimulation-evoked dopamine release (Forster & Blaha, 2003; Lester, Rogers, & Blaha, 2010). All amperometric recordings were made within a Faraday cage to increase signal to noise ratio. A fixed potential (+0.8 V) was applied to the recording electrode, and oxidation current was monitored continuously (10 K samples/sec) with an electrometer (ED401 e-corder 401 and EA162 Picostat, eDAQ Inc., Colorado Springs, CO) filtered at 50 Hz. **A** series of cathodal current pulses was delivered to the stimulating electrode via an optical isolator and programmable pulse generator (Iso-Flex/Master-8; AMPI, Jerusalem, Israel). The stimulation protocol consisted of 20 monophasic 0.5 ms duration pulses (800 μAmps) at 50 Hz every 60 seconds to establish a baseline dopamine response. MFB and DN stimulation-evoked dopamine was monitored for 30 minutes in each mouse. Following these baseline recordings, a random subset of mice received a 1.0 μL infusion (over 1.0 min) of either PBS (control) or the local anesthetic lidocaine (4%) into the DN contralateral to the stimulation site, and dopamine recordings continued for 30 min. Lidocaine blocks sodium channels and has been used during amperometric dopamine

recordings to temporarily block functioning in a local brain site with peak lidocaine responses occurring between 2-5 min post infusion (Lester et al., 2010). At the conclusion of the amperometric recordings, recording electrodes were calibrated in vitro with dopamine solutions (0.2-1.2 μM) administered by a flow injection system (Michael & Wightman, 1999). Thus, change in dopamine oxidation current (nAmp) was converted to dopamine concentration (μM).

Drugs

Urethane (U2500), lidocaine, (L7757), and dopamine (H8502) were obtained from Sigma-Aldrich Chemical (St Louis, MO). Urethane was dissolved in distilled water, and lidocaine and dopamine were dissolved in PBS (ph 7.4).

Histology

Upon the completion of each experimental session, an iron deposit was made in the stimulation site by passing direct anodic current (100 μA and 1 mA, respectively) for 10 sec through the stimulating electrodes, and 1.0 μL cresyl violet stain was infused into the cannula site. Mice were euthanized with a 0.25 ml intracardial injection of urethane (0.345 g/ml). Brains were removed, immersed in 10% buffered formalin containing 0.1% potassium ferricyanide (which causes a redox reaction at the stimulation site resulting in a Prussian blue spot), and then stored in 30% sucrose/10% formalin solution for at least 1 week prior to sectioning. Using a cryostat at -20°C, 30 μm coronal sections were sliced, and electrode placements were determined under a light microscope and recorded on representative coronal diagrams confirming the intended sites were stimulated (Paxinos & Franklin, 2001).

Data Analysis

To quantify MFB and DN stimulation-evoked dopamine efflux, pre-stimulation current values were normalized to zero current values and data points occurring within a range of 0.25 sec pre- and 50 sec post-onset of the stimulation were extracted from the continuous record prior to and following drug infusion. An independent samples t-test was used to assess hemispheric differences in baseline NAc dopamine release. A two-way mixed ANOVA was used to determine the effect of drug infusion (PBS or lidocaine) and time (pre-infusion or 5 min post-infusion) on dopamine release. Dopamine release post infusion was also converted to percent change (with the pre-infusion concentration being 100%), and an independent samples *t*-test was used to determine if there was a significant difference between PBS and lidocaine.

Results

NAc dopamine release following ipsilateral stimulation of the MFB

NAc dopamine release was quantified in each hemisphere as a function of peak height following electrical stimulation of the ipsilateral MFB. No differences were observed between the MFB stimulation-evoked dopamine release in the left NAc (M ± SEM: 1.513 uM ± 0.357) compared to the right NAc (1.614 uM ± 0.466); t (8) = -0.172, p = .867, d = 0.11 (Fig 1).

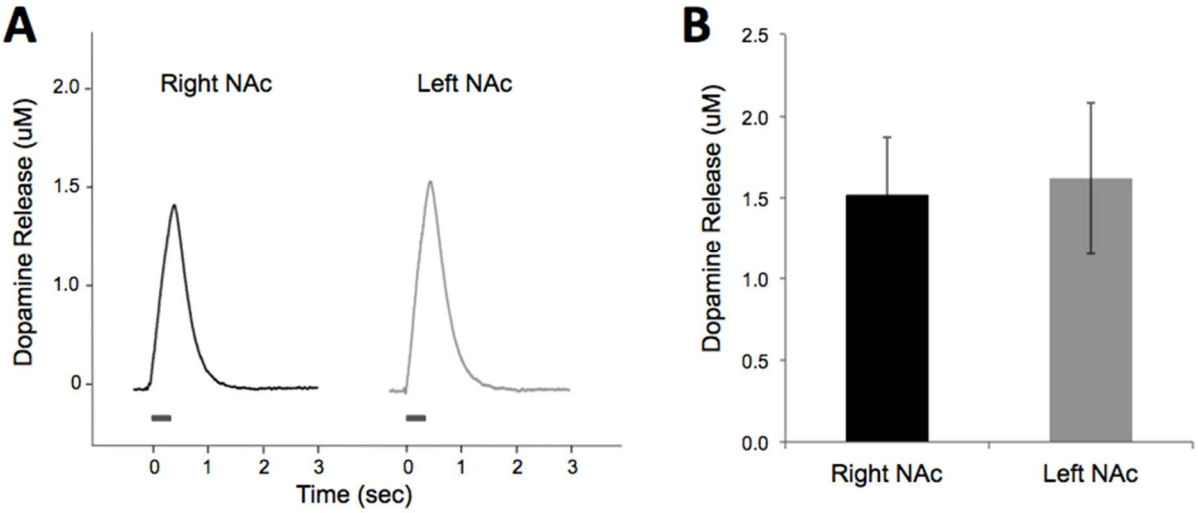

Figure 1. Amperometric recordings of dopamine release in the left or right nucleus accumbens (NAc) in response to electrical stimulation of the ipsilateral medial forebrain bundle (MFB). (A) Profiles illustrate example responses from each recording site. Time zero indicates the start of the train of 20 pulses at 50 Hz. (B) No mean (± SEM) differences in dopamine release were observed between hemispheres.

NAc dopamine release following contralateral stimulation of the cerebellar DN

NAc dopamine release was quantified in each hemisphere as a function of peak height following electrical stimulation of the contralateral DN. DN stimulation-evoked dopamine release was significantly greater in the right NAc (M ± SEM: 0.018 uM ± 0.002) compared to the left NAc (0.011 uM ± 0.001); t (15) = -3.47, p = .003, d = 1.67 (Fig 2).

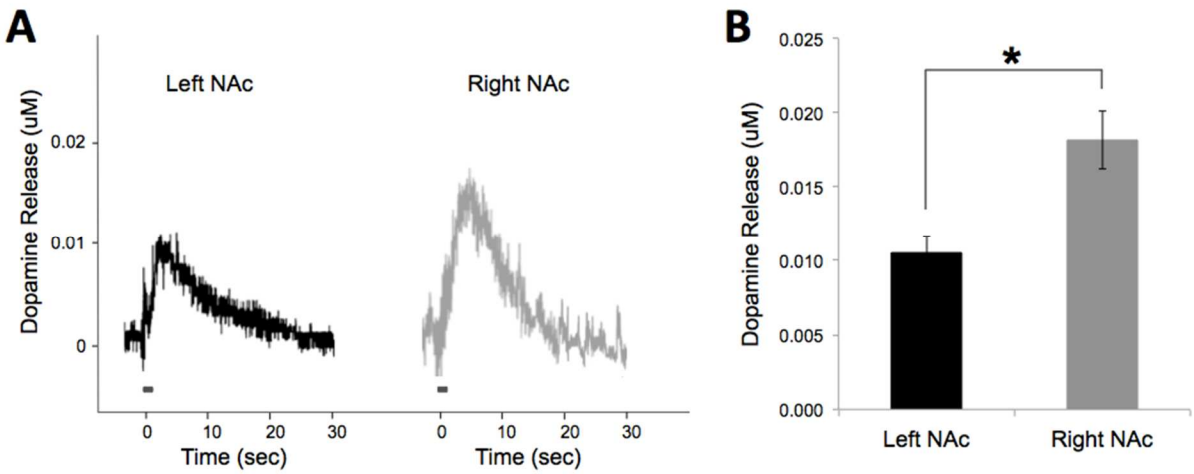

Figure 2. Amperometric recordings of dopamine release in the left or right nucleus accumbens (NAc) in response to electrical stimulation of the contralateral dentate nucleus (DN) of the cerebellum. (A) Profiles illustrate example responses from each recording site. Time zero indicates the start of the train of 20 pulses at 50 Hz. (B) Mean (± SEM) differences in dopamine release were observed between hemispheres. * indicates p = .003

NAc dopamine release following deactivation of the ipsilateral cerebellar DN

During contralateral DN stimulation-evoked dopamine recordings, separate mice received an infusion of either lidocaine or PBS (control) into the ipsilateral DN to determine the impact of hemispheric DN interactions on mesolimbic dopamine transmission. In the left NAc (electrical stimulation in the right DN and infusion into the left DN), a two-way mixed ANOVA revealed no significant interaction between the infusion (PBS or lidocaine) and time (pre-infusion or 5 min post-infusion) on dopamine release, $F(1, 7) = 0.39$, $p = .55$, $\eta_p^2 = 0.05$, and no main effect of infusion on dopamine release, $F(1, 7) = 0.36$, $p = .57$, $\eta_p^2 = 0.05$. Similarly, in the right NAc (electrical stimulation in the left DN and infusion into the right DN), a two-way mixed ANOVA revealed no significant interaction between the drug infusion and time on dopamine release, $F(1, 6) = 0.13$, $p = .73$, $\eta_p^2 = 0.02$, and no main effect of infusion on dopamine

release, $F(1, 6) = 0.01$, $p = .91$, $\eta_p^2 < 0.01$. These results indicate that in both hemispheric NAc recordings, dopamine release was not altered by either infusion (PBS or lidocaine), suggesting DN cross-talk is not significantly influencing NAc dopamine release. Figure 3 shows this data in terms of percent change with dopamine recordings prior to infusion being 100%. Correspondingly, no differences in percent change in dopamine release were observed between lidocaine and PBS infusions in either the left NAc recordings [t (7) = 0.33, $p = .76$, $d = 0.22$, Fig 3A] or right NAc recordings [t (6) = -0.61, $p = .57$, $d = 0.46$, Fig 3B].

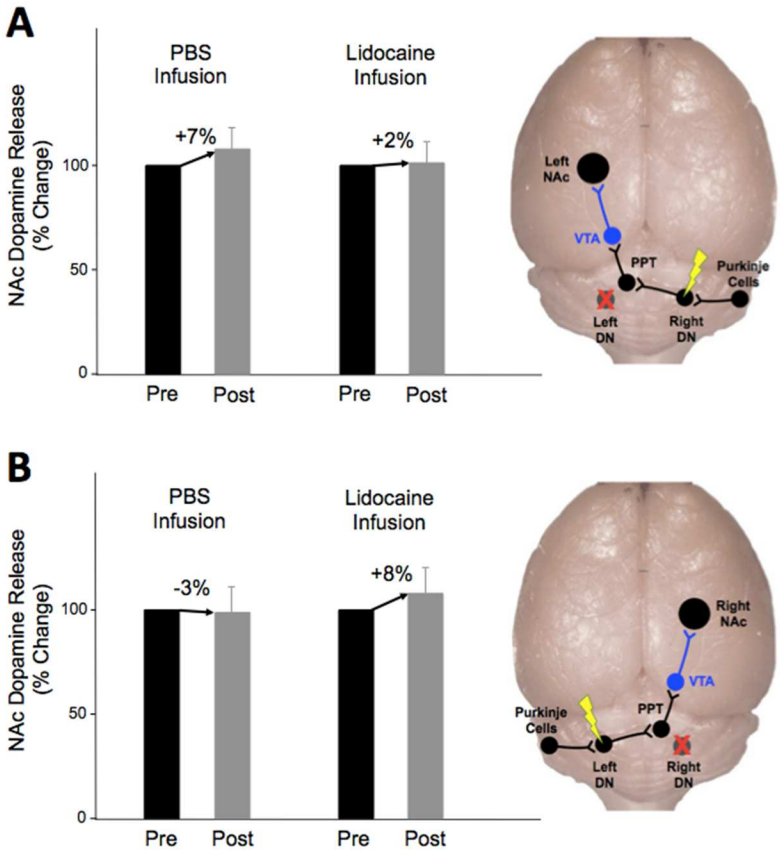

Figure 3. Mean (± SEM) nucleus accumbens (NAc) dopamine release in response to electrical stimulation of the contralateral cerebellar dentate nucleus (DN) pre and post infusion of PBS (control) or lidocaine (4%) in the ipsilateral DN. Neither PBS of lidocaine infusion significantly altered dopamine release in the NAc (A: left, B: right).

Discussion

The current study assessed the hemispheric lateralization of stimulation-evoked dopamine in the NAc and the influence of the cerebellum in regulating this reward-associated pathway. Results suggest that the mesolimbic pathway itself is not responsible for asymmetrical lateralization of dopamine release given NAc dopamine release did not differ between hemispheres when evoked by ipsilateral MFB stimulation. Instead, dopaminergic asymmetry may originate from the cerebellar influence over these pathways. Dopamine release was significantly greater in the right NAc relative to the left when evoked downstream by the DN of the cerebellum. Furthermore, cross-hemispheric talk between the left and right cerebellar DN does not seem to influence mesolimbic dopamine release given that lidocaine infused into the DN opposite the electrically stimulated DN did not alter dopamine release.

Cerebral and cerebellar hemispheres are known to be asymmetrical in structure and function, and many researchers have shown this asymmetry extends to the mesolimbic dopamine system (Hu, Shen, & Zhou, 2008; Hugdahl & Davidson, 2004). Numerous studies using methods of protein analyses in rodents have found greater levels of dopamine and its metabolites in the right NAc relative to the left (Andersen & Teicher, 1999; Budlin et al., 2008; Camp, Robinson, & Becker, 1984; Rosen et al., 1984). Protein analyses such as the ones used in these previous studies are useful in determining dopamine content levels but do not distinguish the neural pathways responsible for modulating dopamine transmission. In vivo experiments have shown the cerebellum is directly involved in regulating dopamine release in the mesocortical system (Mittleman et al. 2008; Rogers et al., 2013). The present study extends these findings by showing

cerebellar output also has the ability to modulate mesolimbic dopamine transmission, with this modulation seemingly contributing to asymmetrically lateralized dopamine release.

Dopamine release in the NAc is known to be associated with reward and motivational processes (Cohen et al., 2012; Matsumoto & Hikosaka, 2009), and some researchers submit that individual differences to natural rewards are a product of asymmetry in the dopamine systems (Tomer et al., 2008). The present results support the notion that reward processes in the brain may be lateralized between cerebrocerebellar networks. This information may have considerable applications for many disorders involving dysfunction of the mesolimbic dopamine system including schizophrenia, Parkinson's, ADHD, and addiction (Davis & Khan, 1991; Dougherty et al., 1999; Kish, Shannak, & Hornykiewicz, 1988). For example, patients with unlilateral onset of Parkinon's disease often develop an asymmetry of dopamine deficiency (Djaldetti, Ziv, & Melamed, 2006; Kempster, Gibb, Stern, & Lees, 1989). In one study, patients whose motor symptoms began on the left side of the body performed more poorly on cognitive tests than those with right-side onset (Tomer, Levin, & Weiner, 1993). These researchers concluded that damage to right-hemisphere dopamine systems plays a greater role in associated cognitive decline than left-hemisphere depletion. Optimal treatment for these symptoms may involve administering different amounts of dopaminergic medication to each hemisphere (Tomer, Aharon-Peretz, & Tsitrinbaum, 2007).

Although the present study is, to the best of our knowledge, the first to reveal cerebellar influence on mesolimbic dopamine asymmetry, many studies support the underlying theory that cerebellar asymmetry covaries with cerebral asymmetry,

especially in abnormal pathology. For example, in stroke patients, cerebellar lateralization switches in parallel with associated cerebral lateralization of language functioning (Connor et al., 2006), and patients with left cerebral hemisphere congenital focal lesions display a reorganized language network associated with the left cerebellum (Lidzba et al., 2008). Furthermore, a positive correlation has been found between the asymmetrical volume of cerebellum and the lateralized volume pattern in the cerebrum (Snyder et al., 1995). These collective findings, along with our study, support the notion that hemispheric lateralization tracks between the cerebellar and cerebral regions.

Conclusions

Previous animal studies have shown greater concentrations of dopamine in the right NAc relative to the left. Although the present study did not find asymmetrical release when directly stimulating the axons of the mesolimbic dopamine system, the results do support lateralization of the mesolimbic dopamine system via modulation of the cerebellar DN. Specifically, stimulation of the left DN leads to greater dopamine release in the right NAc relative to right DN stimulation and left NAc dopamine release. Cerebellar-mediated dopamine pathways have previously been shown to exhibit plasticity and compensatory changes in the neural circuitry of rodent models of autism, providing a potential foundation for the cerebellum to develop unique functional connections between cerebral hemispheres (Rogers et al., 2013). Determining the functional relationship between lateralized cerebrocerebellar networks may lead to novel targets for pharmacological interventions.

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CHAPTER 5: SUMMARY AND CONCLUSIONS

Understanding normal neurochemical dopamine function is necessary not only to identify potential pathology, but also to assess the effectiveness of drugs that might be used for treatment. The nigrostriatal dopamine system consists of dopamine cell bodies in the SNc that project to the dorsal striatum and play a role in the expression of motor processes (Horvitz, 2000; Parent & Hazrati, 1995). The other major dopaminergic circuit, the mesocorticolimbic dopamine system, consists of cell bodies in the VTA that project to the amygdala, NAc, and mPFC (Fibiger & Phillips, 1988; Koob & Swerdlow, 1988). The magnitude and timing of phasic dopamine release is critical for many diverse functions in these output sites including reward, attention, anxiety, and decision-making (Tsai et al., 2009). These differing behavioral processes emerge from variations in dopamine autoreceptor, uptake, and release sites, which lead to site-specific differences in dopamine concentrations during synchronous phasic firing (Venton et al., 2003).

In Chapter 2 we observed and quantified differences in the neurochemical profile of phasic dopamine release in major dopaminergic sites including the striatum, NAc, amygdala, and prefrontal cortex after stimulation of the MFB. We also provided evidence that supported differing behavioral processes in the brain may emerge as a result of spatial and temporal variations in the phasic response. Specifically, these findings indicate that phasic dopamine may have different modes of communication between striatal and corticolimbic regions, with the first being profuse in concentration, rapid, and synaptically confined, and the second being more restricted in concentration but longer-lasting and spatially dispersed. Understanding the various aspects of regional differences

in phasic dopamine transmission may be useful for predicting and manipulating the effects of drugs on dopamine-dependent behaviors.

In Chapter 3 we introduced the notion of a cerebellar influence on the nigrostriatal dopamine system by identifying a pathway originating in the cerebellar dentate that projects to the dorsomedial striatum and examining potential pathology of this pathway in a rodent model of Fragile-X syndrome. Structural abnormalities within the DN of the cerebellum is one of the most common neuronal abnormalities in individuals with ASDs, specifically reduction in Purkinje cells (Hallahan et al., 2009; McKelvey, Lambert, Mottron, & Shevell, 1995; Murakami, Courchesne Haas, Press, & Yueng-Courchesne, 1992). Input from the cerebral cortex to the cerebellum originates from various types of nuclei (e.g., visual, spatial, premotor, motor), but complex synchronization and funneling of information allows Purkinje cells to provide the sole source of output from the cerebellar cortex, via activity on the DN (Voogd & Glickstein, 1998). In this study, we used amperometry to determine if DN-stimulation evoked dopamine response within the dorsomedial striatum is attenuated in mutant Fragile-X mice in comparison to their wildtype controls. Although we found no significant difference of DA release in this area, examination of other basal ganglia circuitry (e.g., nucleus accumbens, substantia nigra, dorsolateral striatum) may reveal neural abnormalities significant to resolving degenerative symptoms seen in ASD.

The findings reported in Chapter 4 further are intended to aid in investigation of cerebrocerebellar networks and examine the possibility of functional asymmetry in the dopaminergic system. In vivo experiments have shown that the cerebellum is directly involved in regulation of the mesolimbic dopamine system by way of connections from

the DN to the VTA (Mittleman et al., 2008; Rogers et al., 2013). Additionally, cerebral and cerebellar hemispheres are known to be asymmetrical in structure and function, and this asymmetry extends to the mesolimbic dopamine system (Hu, Shen, & Zhou, 2008; Hugdahl & Davidson, 2004). We sought to determine if cerebral or cerebellar networks underlie this laterality by measuring stimulation-evoked dopamine release in the NAC through pathways originating in either the cerebellar DN or the cerebral MFB.

We found significant differences in the amplitude of phasic dopamine release in the DN-NAc systems, but not the MFB-NAc pathways. Thus, the results in Chapter 4 support the notion that reward processes in the brain may be lateralized between cerebrocerebellar networks. These results provide insight about the relationship between the cerebrocerebellar networks and lateralization of the dopaminergic system, as well as reveal potentially novel targets for pharmacological interventions in neuropathology of the cerebellum. For example, these results may provide important information relevant to many disorders involving dysfunction of the dopamine system including schizophrenia, Parkinson's, ADHD, and addiction (Davis & Khan, 1991; Dougherty et al., 1999; Kish, Shannak, & Hornykiewicz, 1988). Future studies should target other neural outputs of the dopaminergic system and determine if cerebellar-mediated asymmetry is a prominent feature of these nuclei as well.

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From	Institutional Animal Care and Use Committee
Subject	Animal Research Protocol
Date	June 16, 2016

The institutional Animal Care and Use Committee (IACUC) has taken the following action concerning your Animal Research Protocol No. **0783** Midbrain Dopamine Transmission in Relation to Fragile X Syndrome and Autism

☒ Your protocol is approved for the following period:

From: June 13, 2016 To: June 12, 2018

☐ Your protocol is not approved for the following reasons (see attached memo).

☐ Your protocol is renewed without changes for the following period:

From: _____ To: _____

☐ Your protocol is renewed with the changes described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated _____ for the following period:

From: _____ To: _____

☐ Your protocol is not renewed and the animals have been properly disposed of as described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated _____

Amy L. de Jongh Curry, PhD, Interim Chair of the IACUC

Karyl K. Buddington

Dr. Karyl Buddington, University Veterinarian
And Director of the Animal Care Facilities