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Electrical Stimulation Uses Sodium Channel Dependent Depolarization To Produce Exocytotic-Like Dopamine Release And Rotational Behavior In Vivo

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Dedicated to Baby G, the most important person in my life that I have not met

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by

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Abstract

The goal of this project was to establish Electrical Stimulation (ES) in combination with *In Vivo* Intracerebral Microdialysis (IVMCD) as a methodology to evoke exocytotic-like dopamine (DA) release. To provide evidence that ES mimics action potential-mediated DA exocytosis, we hypothesized that ES produces depolarization of the membrane potential that is dependent upon sodium (Na^+) -channels to produce DA release concomitantly with rotational behavior. To test this, rats received electrode and cannulae implants along the medial forebrain bundle, which contains the DAergic nigrostriatal pathway, and a microdialysis probe at the striatum to undergo IVMCD testing. To begin, steady baseline DA levels were assessed followed by ES, subsequent post ES samples were collected, followed by an infusion of Na^+ channel blocker, lidocaine. Next, a second phase of ES was applied to assess the effects of lidocaine on ES-evoked DA overflow, and two additional post ES samples followed. The data indicate that lidocaine decreased both basal and electrically stimulated DA release, and reduced associated rotational behavior. The current data support the idea that ES activates Na^+ channels to induce exocytotic-like DA release and rotational behavior. These data validate ES in combination with IVMCD as an effective methodology to study plasticity of exocytotic mechanisms that alter DA neurotransmission. Specifically, future research that aims to understand how DA neurotransmission is altered in behavioral disorders, such as neurodegenerative or substance use disorders, can utilize this innovative combination of ES and IVMCD.

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Introduction

History of Electrical Stimulation

Electrical stimulation (ES) has played an important role in elucidating how electrical currents are important in the physiological function of neurons as well as the functional organization of the nervous system. The role of electricity in biological systems was formally investigated for the first time in the 18th century when Luigi Galvani discovered that muscle movements could be evoked when applying electrical current to muscle tissue of the Peripheral Nervous System (Piccolino, 1997). This discovery helped to conceptualize that a cell has the capability to generate its own electrical energy. It was not until the late 19th century when Fritsch and Hitzig revealed that the Central Nervous System was electrically excitable. Using ES, they discovered that the cortex was important for producing behavior, and that different behaviors were localized in different locations of the cortex (Fritsch & Hitzig, 1870).

ES has been a valuable tool to map other brain functions beyond the initial findings by Fritsch and Hitzig. For example, a topographical representation of the cortex was developed based on electrically evoked sensory and motor behavior (Penfield & Jasper, 1955; Woolsey, 1979). ES of the lateral hypothalamus has also been shown to evoke motivated feeding behavior (Hoebel & Teitelbaum, 1962; Berridge & Valenstein, 1991) and to discover that other limbic structures, such as the septal nucleus, modulate reinforcement (Olds & Milner, 1954). Therefore, ES of brain circuits has been an important tool in neuroscience to elucidate the behavioral organization of the Central Nervous System.

Thus, ES has been used to explore functional circuits by correlating evoked behaviors with stimulated brain sites. Moreover, the ability to study the effects of ES at the neuronal level

has also been a valuable tool in the history of neuroscience. In 1952 the action potential was characterized using ES to demonstrate how ion permeability changes with depolarization of the membrane potential (Hodgkin & Huxley 1952). Another *in vitro* study utilized ES to provide evidence that inward calcium (Ca^{++}) current is needed for synaptic signaling upon depolarization (Llinas et al., 1981). Due to the parallels between ES and endogenous mechanisms of release, this tool has been utilized *in vitro* to study neurotransmission. For example, ES has been used to show that the availability of precursor tyrosine can change the magnitude of dopamine (DA) release (Milner & Wurtman, 1984). ES is now a traditional tool in neuroscience and is commonly used *in vitro* and *in vivo* to study neural mechanisms and behavior (Reynolds & Wickens, 2000).

Neurotransmission

All behavior is dependent upon neurotransmission, or neuronal communication, that is comprised of electrophysiological activity that results in neurotransmitter release. One critical electrophysiological signal is the action potential: This occurs when the resting membrane potential depolarizes to a critical threshold and voltage-gated sodium (Na^+) channels open. This event triggers a change in the permeability of the membrane allowing extracellular Na^+ to rush into the neuron. This movement of positively charged Na^+ ions peaks and Na^+ channels become refractory. The depolarizing changes in the membrane potential subsequently actives voltage-gated potassium (K^+) channels to open. Electrostatic pressure causes K^+ ions to rush out, resulting in hyperpolarization back towards the resting membrane potential. To terminate the action potential, there is a refractory period during which a subsequent action potential cannot be generated. During this time, the resting membrane potential is re-established by extracellular K^+ diffusion away from the local site and a Na^+/K^+ pump. The action potential propagates towards

the presynaptic terminal and in response to the depolarizing effects of the action potential, voltage-dependent Ca^{++} channels open, allowing Ca^{++} ions to flux inward. Intracellular Ca^{++} causes vesicles to dock onto the presynaptic membrane (Söllner et al., 1993). *Exocytosis* culminates with neurotransmitter released from the vesicles into the synaptic cleft, the hallmark of neurotransmission.

A technique that is meant to mimic exocytosis should demonstrate, in part, the three critical characteristics of action potential-mediated neurotransmitter release: 1) Na^{+} channel-driven depolarization of the membrane potential, 2) opening of voltage dependent Ca^{++} channels allowing the influx of Ca^{++} into the terminal bouton, 3) and vesicular based release of the neurotransmitter into the synapse. Therefore, demonstration that a technique, such as ES, produces exocytotic-like release increases the interpretability of experimental data reflecting changes in neurotransmission. This thesis begins with a brief review of techniques used to study DA neurotransmission.

Amphetamine Evoked DA Overflow

Amphetamine has often been used to measure the functional status of DA systems, either to evoke behavior (Essman et al., 1992) or as a functional index of DA "releasability" (Robinson et al., 1994). However, amphetamine does not work by exocytotic mechanisms but instead produces increases in extracellular DA by an exchange/diffusion process (Fischer & Cho, 1979; Parker & Cubeddu, 1986; Levi & Raiteri, 1993; Raiteri et al., 1979). In exchange for amphetamine entry, the DA transporter transfers DA from the inner cytoplasmic pool out to the extracellular environment. Therefore, despite its ability to increase extracellular DA, amphetamine is not a desirable tool to understand changes in mechanisms of exocytosis.

Potassium K^+ Evoked DA Overflow

K^+ stimulation works by depolarizing the membrane, and triggering action potentials that induce DA release (Stanford et al., 2000). K^+ stimulation has been used to investigate changes in exocytosis following neurotransmitter-depleting lesions (Abercrombie & Zigmond, 1989; Lindefors et al., 1989). These studies have provided important insights about changes in depolarization-based release. Nonetheless, a limitation of K^+ stimulation by reverse dialysis is that minimal behavior is displayed upon stimulation (Casanova et al., 2013; Tran-Nguyen et al., 1996) and the pharmacokinetics of K^+ cannot be precisely controlled to mimic the sub-second timeframe of action potentials. Thus, a better approach is required that addresses the weaknesses presented by amphetamine and depolarizing stimulation using K^+.

Optogenetic Evoked DA Overflow

Optical stimulation works by activating transgenic light-sensitive receptors such as channelrhodopsin to trigger neurotransmitter release (Deisseroth, 2011). Optogenetics can target specific neurotransmitter systems. For example, anatomically precise targeting of DA cell bodies of the substantia nigra increases confidence for DA-specific effects (Bass et al., 2010), which is not possible with amphetamine, K^+ stimulation, or ES. *In vitro* and *in vivo* studies have validated optically induced release as comparable to exocytosis, especially because these light sensitive receptors can be programmed to express cation channels that naturally trigger depolarization via transgenic Na^+ channels or vesicular docking via transgenic Ca^{++} channels (Izquierdo-Serra et al., 2013). Although this technique is more powerful than ES due to specificity, it poses challenges in studying endogenous receptors. Because optical stimulation exclusively activates light

sensitive receptors, this poses challenges in studying changes in endogenous receptors that modulate neurotransmission.

ES-Evoked DA Overflow

ES is a powerful tool to investigate changes in neurotransmission for four reasons: 1) ES evokes behavior largely related to the function of the locus being stimulated. Specifically, unilateral ES of the nigrostriatal pathway produces motor behavior in the form of rotational behavior (Pycock, 1980). 2) Behavioral and neurophysiological responses to ES are intensity-dependent (Castañeda et al., 1985) and frequency-dependent (Castañeda, unpublished data). 3) ES can be precisely turned on and off; thus avoiding the long-enduring pharmacokinetics of drug stimulation. 4) Lastly, ES activates exocytotic events to produce neurotransmitter release *in vitro* (Mulder 1983). These features are important criteria necessary to gain confidence that ES produces a physiologically relevant response when evoking DA release. However validation that electrically induced DA release mimics exocytosis in awake and freely moving animals is yet to be established.

In Vivo Intracerebral Microdialysis (IVMCD) of DA Overflow

Coupled with ES, IVMCD is a powerful choice to study changes in neurotransmission for four reasons: 1) this technique allows for the sampling of extracellular DA levels while leaving the brain functionally intact and without disrupting feedback mechanisms from other brain structures. For example, release can be augmented or attenuated by an intact network circuitry in a whole animal preparation, in contrast to isolated brain fragments typical of *in vitro* procedures. Therefore, IVMCD allows for a more physiologically relevant assessment of neuronal function. 2) IVMCD yields DA samples that can be accurately quantified using biochemical techniques

such as high pressure liquid chromatography coupled with electrochemical detection. 3) Repeated sampling from an animal can continue across extended time periods to gain insight into the dynamics of DA exocytosis. 4) Lastly, this technique is most powerful for its ability to estimate the dynamics of exocytosis *simultaneously* with ongoing behavior in an awake animal preparation.

ES in Combination with IVMCD

The aim of this thesis is to validate ES in combination with IVMCD as a method to study exocytotic-like neurotransmitter release concomitantly with an evoked behavioral correlate. Validation requires the demonstration that modulatory mechanisms of exocytosis are necessary to evoke the neurochemical and behavioral responses produced by ES. These include: intensity- and frequency-dependent responses to ES, Na^{+} channel-dependent activation, dependence upon Ca^{++} availability, Ca^{++} channel sensitivity, as well as other criteria such as vesicular based release of the neurotransmitter.

Preliminary unpublished evidence in the laboratory of Dr. Castañeda has already shown that ES produces intensity-, frequency-, and Ca^{++}-dependent responses akin to exocytosis. For example, these data show that the Ca^{++} channel blocker verapamil attenuates ES-evoked DA overflow. Also, the Ca^{++} chelater ethyleneglycol-bis 2 amino-ethylether-N,N,N,N-tetra acetic acid (EGTA) attenuates ES-evoked DA overflow. The present thesis builds upon this evidence with converging data that ES, *in vivo*, is dependent upon Na^{+} channel driven depolarization.

Global Hypothesis

The global hypothesis is that ES activates Na^+ channel dependent depolarization akin to action potentials that subsequently produces DA exocytosis. Our working hypothesis is that Na^+ channel blockade attenuates electrically induced DA overflow and rotational behavior.

Specific Aims

Specific Aim 1. To determine whether electrically stimulated DA overflow is Na^+ channel dependent

Specific Aim 2. To determine whether electrically stimulated rotational behavior (ESRB) is Na^+ channel dependent

To accomplish these specific aims, the Na^+ channel blocker lidocaine was infused into the medial forebrain bundle (MFB) of the nigrostriatal pathway and electrically evoked striatal DA overflow, measured by IVMCD, and ESRB were quantified. It was predicted that blocking Na^+ channels during ES would attenuate DA overflow and ESRB. Figure 1. outlines the experimental procedures used to address specific aims 1 and 2.

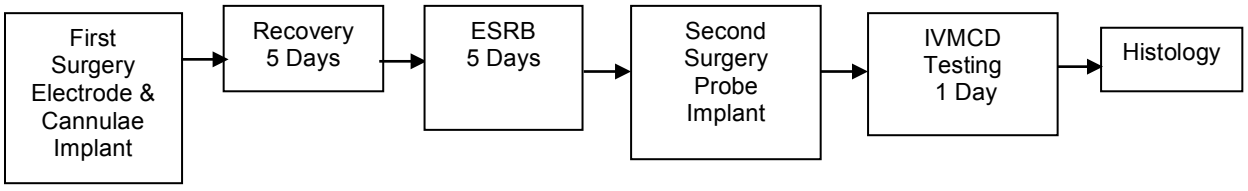

Figure 1. Experimental Procedures

Methods

Subjects

Male and female Wistar rats weighing 200-500 gm were maintained in 36 X 39 X 19 cm plastic housing at the vivarium in The University of Texas at El Paso (UTEP) Psychology department at (Tecniplast) under a reversed 12:12 light/dark cycle (lights off at the 0830). The UTEP Animal Care Program provided husbandry. Food and water were made available ad lib during the entire course of the study except during testing. Immediately before experimentation, all rats were habituated in the testing room by daily handling for 5 days (5-10 min). The UTEP Institutional Animal Care and Use Committee approved the methodology described herein. Nine animals completed the following methodological stages of the experiment. Additional animals were excluded from this study for the following reasons: surgical complications, failure to meet ESRB criterion, probe failure, and unsecure implants.

First Surgery

Using standard stereotaxic procedures with coordinates by Paxinos & Watson (2006), with bregma and lambda horizontal to each other, bilateral holes were drilled through the exposed skull at +0.5 mm anterior to bregma and ±2.5 mm lateral aimed at the caudate nucleus for future implantation of a microdialysis probe in the second surgery (described below). Next, bipolar twisted wire stainless steel electrodes (Plastics One MS303/3, 0.150 mm diameter) were implanted bilaterally aimed at the MFB (coordinates in mm from bregma: A -4.4, L ±1.2, V 8.7). Stainless steel guide cannulae (23 ga) were also implanted bilaterally aimed at the MFB, at the level of the lateral hypothalamus (coordinates from bregma in mm A–2.5, L ±1.8, V -5.0). Later, during IVMCD testing, an infusion cannula was inserted through this guide cannula to the depth

of the MFB at V 8.4 mm (see IVMCD testing). All implants were secured to the skull with jeweler's screws tightened into trephines and dental cement. Finally, the scalp was sutured and dabbed with antibiotic cream.

A Priori Criterion: ESRB

Five days after stereotaxic surgery animals were tested for ESRB before proceeding to IVMCD testing. For five consecutive days, ES was administered in increasing intensities (50-300 μA in 50 μA intervals) to determine intensity dependence of ESRB. To be included in the experiment, animals had to satisfy an a priori criterion of at least 8 quarter turns/10 sec at 300 μA. However, 2 of the 9 animals were stimulated at 200 μA to prevent tumbling associated with higher intensities. ES was delivered by a Grass S9 Stimulator in series with a Grass Constant Current Unit. ES consisted of monophasic rectangular pulses at 50 Hz, 0.1 ms pulse duration, 10-sec train duration, and a 2-min inter-train interval. Each electrode implant was tested using normal and reversed current flow to determine optimal testing conditions. Essentially, this a priori testing provided behavioral validation of precise electrode placement into the MFB.

Microdialysis Probe Construction

Microdialysis probes were of concentric design with a 4 mm length effective area of dialysis (Robinson & Whishaw, 1988). To construct these, cellulose (dialysis membrane) was cut to a length of 10 mm, and inserted into the edge of a 26 ga 13 mm-length stainless steel cannula, which was previously acid etched. The dialysis membrane was then glued onto the stainless steel cannula with 2 ton epoxy (Devcon) and left to dry for a few hours. Next the dialysis membrane was cut to a length of 4.25 mm; then 0.25 mm of the tip was sealed with 2 ton epoxy and left to dry overnight. The next day, the outlet PE-20 tubing was pierced 8 mm from one end with a 30

ga needle and the silica inlet tubing threaded through this piercing. The outlet tubing pressure fits over the stainless steel cannula and is pushed over the cannula to a length of 5 mm, leaving a 3 mm space between the end of the stainless steel cannula and the entry-point of the silica tubing. The silica inlet tubing was then pushed through the lumen of the stainless steel cannula and through the lumen of the dialysis membrane until a 0.25 mm gap was reached between the end of the silica tubing and the epoxy plug. Finally, the junction between the outlet tubing and the stainless steel cannula, including the section where the inlet tubing pierces to enter into the probe assembly, was placed inside a pipette tip, and subsequently filled with 2 ton epoxy.

Efficacy of Microdialysis Probe In Vitro Recovery

In vitro testing was conducted to determine the efficacy of microdialysis probes to collect DA and its major metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), and the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA). An artificial cerebrospinal fluid (aCSF, modified Ringer's buffer medium) containing 128.3 mM Na^{+}, 2.68 mM K^{+}, 1.35 mM Ca^{++}, 2.0 mM Mg^{++}, and 2.0 mM $PO4^{+}$ was used as dialyzing media at 37^{o} C.

At least one day before *in vitro* recovery, all test probes were flushed thoroughly. The flushing procedure consisted of pumping HPLC water through dialysis probes placed in a solution of 70% ethanol for 30 minutes at a rate of 5.0 µl/min. The ethanol was then replaced with HPLC grade water and the flow rate was changed to 0.15 µl/min and left to pump overnight. The next day microdialysis probes were filled with aCSF. At this point, the microinfusion pump was set to 1.5 µl/min and maintained at this rate throughout the recovery procedure; the same rate that was used during *in vivo* testing. After 15 min, the probes were immersed into aCSF containing 500 pg/µl of DA and 200 pg/µl for each of DOPAC, HVA, and

5-HIAA ("sampling standard") for the purpose of calculating a recovery coefficient. The probes were then allowed to equilibrate to test conditions for 20 min. Next, two 10 min samples were collected. For quantitation, a 25% dilution of the sampling standard was prepared for analysis of the *in vitro* recovery samples; this is within the range of recovery for microdialysis probes with a 4 mm cellulose dialyzing membrane. High performance liquid chromatography coupled with electrochemical detection (HPLC/EC) was used to measure monoamines (see below). Peak heights from the standard and samples were used to calculate the percent recovery of probes. *In vitro* recovery estimates were used to correct for probe efficiency during in vivo procedures, although it is acknowledged that *in vitro* conditions do not mimic the "tortuosity" of the *in vivo* milieu (Benveniste et al., 1989).

Second Surgery - Microdialysis Probe Implantation

Rats that met the a priori criterion for reliable ESRB underwent a second stereotaxic surgery to implant a microdialysis probe into the caudate nucleus (flat skull, from bregma A +0.5, L ± 2.5, V -7.0) ipsilateral to the effective electrode. The stereotaxic surgery proceeded as described for electrode implantation except that a microdialysis probe was lowered into the caudate nucleus region, where DA is released. When this surgery was complete, rats had fluid lines, for perfusing the microdialysis probe, and electrical wiring, connected to the stimulating electrode, emanating from a dental acrylic pedestal and were placed into a testing chamber (30 X 35 cm) to recover from the surgery overnight. Microdialysis testing took place the next day.

IVMCD

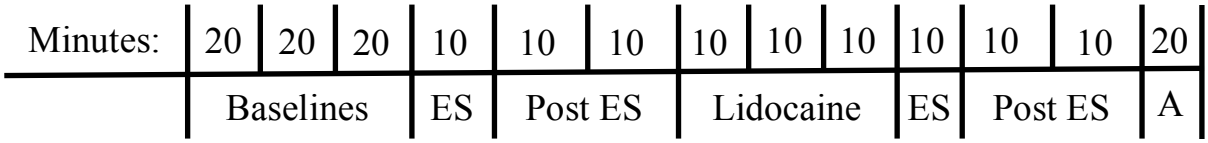

Figure 2. IVMCD Timeline

Experiment (Aim 1 & Aim 2)

Figure 2 outlines the test conditions and timeline for IVMCD. To begin, 20-min samples were collected to establish baseline levels of DA overflow. Baseline samples were collected until 3 samples varied <10% from each, usually 3-5 sample collections.

Next, unilateral ES (monophasic rectangular pulses, 0.1 msec pulse duration, 50 Hz) was applied for 10 sec every 2 min during a 10-min sampling bin. The current applied was 300 μA, the highest intensity used during a priori procedures. ESRB was recorded during ES only.

Post ES, two 10-min samples were collected to reestablish baseline.

Next all animals received lidocaine treatment (4% lidocaine at 0.5 μl/min infusion rate over 12 min). The microinfusion took place via a 30 ga microinfusion cannula inserted 8.4 mm below skull surface through the previously implanted guide cannula, thus targeting the MFB. Three 10-min samples were collected thereafter to establish a steady-state baseline under lidocaine conditions.

ES was applied as during the previous control conditions and ESRB was counted.

Subsequently, two 10-min post ES samples were collected.

To serve as a positive control, 1.5 mg/kg/ml of amphetamine (A) was administered intraperitoneally to verify the animal preparation was viable. One 20-min sample was collected

immediately thereafter. Dialysate samples were quantified using HPLC/EC based on standards injected into the system.

HPLC/EC Methodology

HPLC/EC methodology consists of two phases for analysis - separation and detection, which are used to assay for brain DA, its major metabolites DOPAC and HVA, and the serotonin metabolite 5-HIAA.

Reverse Phase Chromatography

Standards and dialysate samples were injected onto an HPLC system via a Rheodyne Model 7125 manual injector. The system consisted of an ESA Model 584 dual piston pump set at a rate of 0.5 ml/min in continuity with a pulse dampener. The stationary phase of the HPLC was a 15 cm length Dynamax reverse-phase column (C-18; 8 μm particles, 0.46 cm i.d.; Varian Inc). The mobile-phase consisted of 60 mM sodium phosphate, 30 mM citric acid, 0.1 mM EDTA, 20mg/liter sodium dodecyl sulfate and 20% methanol at a pH of 3.75. The order of elution was DOPAC, 5-HIAA, HVA, and DA and the run time of approximately 10 min.

Electrochemical Detection

To decrease background noise, the samples were passed through a conditioning cell (ESA Model 5021) set to oxidize at +100mV. Next, a High Sensitivity Analytical Cell (ESA Model 5010) was used with the first electrode set to oxidize at +340 mV (500 nA sensitivity) and the second electrode was set to reduce at -340 mV (5 nA sensitivity). A Coulochem II detector (Model 5200A) was used to measure the amount of analytes based on the peak height generated. The chromatographs were obtained by EZChrom SI software (Agilent Technologies).

Histology

Animals underwent post mortem intracardiac perfusion. First, buffered saline was perfused to remove blood followed by 4% formaldehyde in buffered saline to fixate the brain. The brain was then removed from the skull and placed into 30% sucrose paraformaldehyde. Once the brain was submerged, the solution was replaced until the brain sunk again. The brain was then sectioned in a coronal plane on a Leica CM 1850 cryostat. Brain slices were stained with cresyl violet and examined for placement of electrodes, infusion cannulae, and microdialysis probes.

Criteria for accurate placement of probes included those that were within the corpus of the striatum. Criteria for accurate placement of infusion cannulae included those that extended at least 2 mm above the MFB for a sufficient sphere of lidocaine diffusion. All animals that met the a priori behavioral criterion for ESRB for 8 turns/10 sec train were included and examined for electrode placement at the MFB.

Statistical Analysis

Statistics were conducted using Prism™ (GraphPad software v6). For DA, DOPAC, HVA and 5HIAA one way repeated measures ANOVAs were conducted across the three time bins sampled during baselines before and after lidocaine infusion; then two-way repeated measures ANOVAs were conducted to examine the effects of ES and lidocaine. When a main effect for repeated measures was found, a follow up one-way repeated measures ANOVA was conducted for each phase of lidocaine treatment (before, after). The Geisser-Greenhouse correction was used when sphericity was violated (i.e., epsilon < 1.0). When significant main effects were found, post-hoc Sidak and Tukey tests were used for pair-wise comparisons. For

ESRB, a two-way repeated measures ANOVA was used to evaluate the effects of lidocaine during the five trains of ES.

Results

Effect of Lidocaine on Electrically Stimulated DA Overflow (Figure 3A):

A one-way repeated measures ANOVA was conducted for the 3 baseline samples collected before ES (data not shown). There was no change across time before lidocaine ($F_{1.59, 12.72} = 0.30$, $p > 0.05$) or after lidocaine ($F_{1.33, 10.61} = 0.08$, $p > 0.05$). Therefore, the average of these baseline intervals was used in subsequent analyses.

Figure 3A illustrates DA levels within the time course of ES before lidocaine and after lidocaine microinfusion. A 2-way (lidocaine, time) repeated measures ANOVA was conducted. A significant effect of lidocaine treatment was found ($F_{1, 8} = 17.56$, $p<0.05$), indicating that in general lidocaine decreased DA levels. During baseline, a post-hoc test revealed a significant decrease in DA levels in response to lidocaine treatment compared to control conditions (Sidak, $p<0.05$). During ES, there

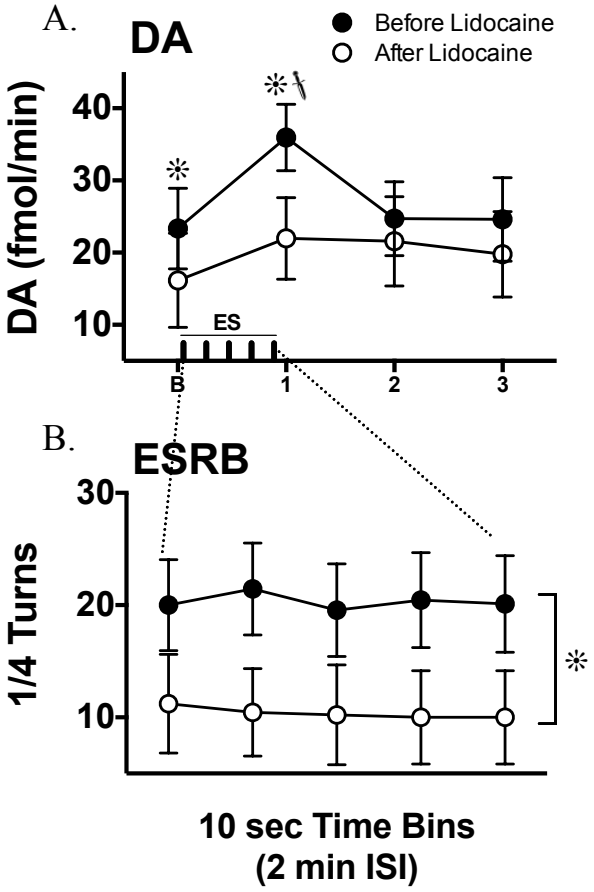

Figure 3. Effect of Lidocaine on A) Electrically Evoked DA, and B) ESRB

A) Extracellular levels of DA (fmol/min) were measured during baseline (B), a 10-min collection interval (1) during which ES (**I**) was applied (5 X 10-sec trains, 2-min ISI), and two additional 10-min intervals (2,3). Animals were tested under control conditions (before lidocaine infusion, ●) and following lidocaine treatment (○). After lidocaine infusion, there was a decrease in baseline (B) DA levels compared to control conditions (⁕, $p< 0.05$). Extracellular levels of DA during ES were also significantly reduced by lidocaine compared to control conditions (⁕, $p< 0.05$). ES increased extracellular levels of DA during control conditions (↟ , $p< 0.05$). Immediately after ES, DA levels returned to baseline levels (p's>0.05). In the lidocaine condition, there were no significant differences across time.

B) ESRB was measured in quarter turns during the 10-min interval in which ES was applied in five 10-sec trains (2-min ISI). Prior to lidocaine (●) all animals demonstrated vigorous contraversive turning in response to ES. After lidocaine (○) there was a significant reduction in turning compared to turning during control conditions (⁕, $p< 0.05$). In both conditions, the rate of turning was sustained across the 5 trains of ES ($p>0.05$).

was also a significant decrease in extracellular DA levels under lidocaine treatment compared to control conditions (Sidak, $p<0.05$).

A significant main effect of repeated measures across time was found ($F_{3, 24} = 12.40$, $p< 0.05$). A follow-up 1-way repeated measures ANOVA conducted during the control phase (before lidocaine treatment) revealed a significant change in DA across time ($F_{2.07, 16.57} = 11.50$, $p< 0.05$). Post-hoc pairwise comparisons revealed a significant increase in extracellular DA levels during ES compared to all other timepoints (Tukey, p's<0.05). A 1-way repeated measures ANOVA for the lidocaine phase was not significant ($F_{1.54, 12.32} = 3.01$, $p>0.05$). A significant interaction (lidocaine X time) was found ($F_{3, 24} = 3.90$, $p< 0.05$) indicating that extracellular DA levels between control versus lidocaine treatment varied as a function of time.

Effect of Lidocaine on ESRB (Figure 3B):

Figure 3B illustrates ESRB before lidocaine and after lidocaine microinfusion. A 2-way (lidocaine, ES) repeated measures ANOVA was conducted. A significant main effect of lidocaine treatment was found ($F_{1, 8} = 7.74$, $p< 0.05$) indicating that in general lidocaine decreased ESRB. During every ES train, post-hoc tests revealed a significant decrease in ESRB in response to lidocaine treatment compared to control conditions (Sidak, p's<0.05). There was no significant main effect for ES ($F_{4, 32} = 0.72$, $p>0.05$), indicating that ESRB remained consistent throughout the five ES time bins. There was no significant interaction (lidocaine X ES) found ($F_{4, 32} = 1.16$ $p>0.05$).

Effect of Lidocaine on ES-Evoked DOPAC Overflow (Figure 4A):

Figure 4A illustrates DOPAC levels within the time course of ES before lidocaine and after lidocaine microinfusion. A 2-way (lidocaine, time) repeated measures ANOVA was conducted. A significant effect of lidocaine treatment was found ($F_{1, 8} = 9.13$, $p < 0.05$) indicating that in general lidocaine increased DOPAC levels. During ES, a post-hoc test revealed a significant increase in DOPAC levels in response to lidocaine treatment compared to control conditions (Sidak, $p<0.05$). During both post ES samples, there was also a

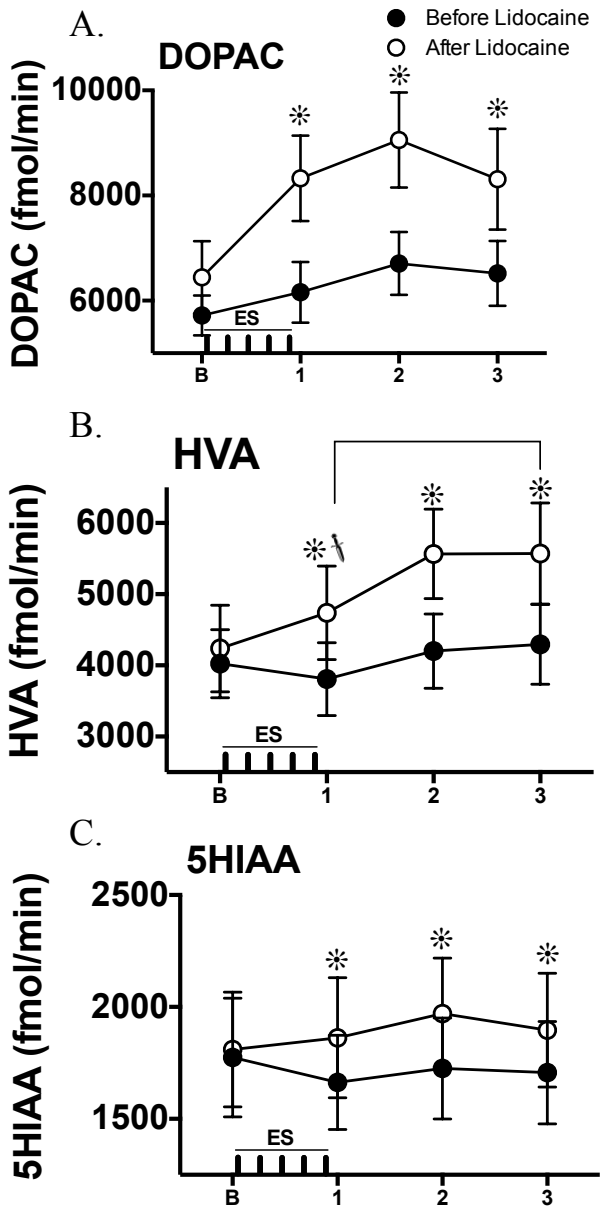

Figure 4. Effect of Lidocaine on ES-Evoked Metabolite Overflow A) DOPAC, B) HVA, C) 5HIAA

Extracellular levels of monoamine metabolites (fmol/min) were measured during baseline (B), a 10-min collection interval (1) during which ES (▮) was applied (5 X 10-sec trains, 2-min ISI), and two additional 10-min intervals (2, 3). Animals were tested under control conditions (before lidocaine infusion, ●) and following lidocaine treatment (○).
A) DOPAC: After lidocaine infusion, there was an increase in DOPAC levels during ES compared to control conditions (✳, $p< 0.05$). Under lidocaine condition, extracellular levels of DOPAC during post ES samples (2 & 3) remained significantly increased (✳, p's< 0.05). ES increased extracellular levels of DOPAC based on the results of the 2-way ANOVA repeated measures factor ($p<0.05$); but neither treatment factor alone yielded a significant change across time (p's=0.07).
B) HVA: After lidocaine infusion, there was an increase in HVA levels during ES compared to control conditions (✳, $p< 0.05$). Under lidocaine condition, extracellular levels of HVA during post ES samples (2 & 3) remained significantly increased (✳, p's< 0.05). In contrast to ES, HVA levels increased during the last post ES sample (3) (ϟ , $p<0.05$).
C) 5HIAA: After lidocaine infusion, there was an increase in 5-HIAA levels during ES compared to control conditions (✳, $p< 0.05$). Under the lidocaine condition, extracellular levels of 5-HIAA during post ES samples (2 & 3) remained significantly increased (✳, p's< 0.05).

significant increase in extracellular DOPAC levels under lidocaine treatment compared to control conditions (Sidak, p's<0.05).

A significant main effect of repeated measures was found ($F_{3, 24} = 7.05$, $p < 0.05$) indicating a general increase in DOPAC levels across time. Follow-up 1-way repeated measures ANOVAs for each treatment condition, before and after lidocaine, revealed no significant changes in DOPAC levels across time. There was no significant interaction (lidocaine X time) found ($F_{3, 24} = 1.28$, $p > 0.05$).

Effect of Lidocaine on ES-Evoked HVA Overflow (Figure 4B):

Figure 4B illustrates HVA levels within the time course of ES before lidocaine and after lidocaine microinfusion. A 2-way (lidocaine, time) repeated measures ANOVA was conducted. A significant effect of lidocaine treatment was found ($F_{1, 8} = 16.04$, $p < 0.05$) indicating that in general lidocaine increased HVA levels. During ES, a post-hoc test revealed a significant increase in HVA levels in response to lidocaine treatment compared to control conditions (Sidak, $p < 0.05$). During both post ES samples, there was also a significant increase in extracellular HVA levels under lidocaine treatment compared to control conditions (Sidak, p's<0.05).

A significant main effect of repeated measures across time was found ($F_{3, 24} = 8.26$, $p < 0.05$). A follow-up 1-way repeated measures ANOVA conducted during the control phase (before lidocaine treatment) revealed a significant change in HVA across time ($F_{2.17, 17.36} = 5.12$, $p < 0.05$). Post-hoc pairwise comparisons revealed a significant increase in extracellular HVA levels during the last post ES sample compared to ES (Tukey, $p < 0.05$). A 1-way repeated measures ANOVA conducted during the treatment phase (after lidocaine treatment) revealed a significant change in HVA across time ($F_{1.50, 11.97} = 6.15$, $p < 0.05$) although there were no

significant differences in post-hoc pairwise comparisons. A significant interaction (lidocaine X time) was found ($F_{3, 24} = 3.61$, $p < 0.05$) indicating that extracellular HVA levels between control versus lidocaine treatment varied as a function of time.

Effect of Lidocaine on ES-Evoked 5HIAA Overflow (Figure 4C):

Figure 4C illustrates 5HIAA levels within the time course of ES before lidocaine and after lidocaine microinfusion. A 2-way (lidocaine, time) repeated measures ANOVA was conducted. A significant effect of lidocaine treatment was found ($F_{1, 8} = 15.01$, $p<0.05$) indicating that in general lidocaine increased 5HIAA levels. During ES, a post-hoc test revealed a significant increase in 5HIAA levels in response to lidocaine treatment compared to control conditions (Sidak, $p<0.05$). During both post ES samples, there was also a significant increase in extracellular 5HIAA levels under lidocaine treatment compared to control conditions (Sidak, p's<0.05).

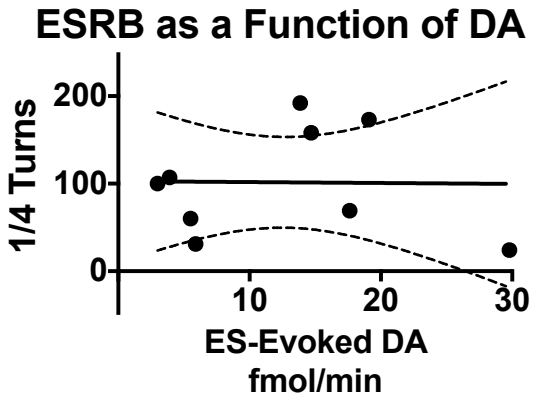

Figure 5. Linear regression analysis of ESRB in relation to ES-Evoked DA Overflow.

Only data prior to lidocaine infusion (●) were used to display the relationship between ESRB as a function of ES-evoked DA. There was no significant correlation between ES-evoked DA overflow and turning ($r=-0.01$, $p>0.05$).

There was no significant main effect of repeated measures across time ($F_{3, 24} = 1.36$) $p>0.05$). However, a significant interaction (lidocaine X time) was found ($F_{3, 24} = 3.39$, $p < 0.05$) indicating that extracellular 5HIAA levels between control versus lidocaine treatment varied as a function of time.

Correlation between Electrically Stimulated Rotational Behavior and DA Overflow (Figure 5): Figure 5 illustrates ESRB as a function of ES-evoked DA levels. ES-evoked DA levels were calculated as the difference in the amount of extracellular DA evoked by ES from baseline levels. These data produced a Pearson correlation coefficient of -0.01, which was not statistically significant ($p>0.05$).

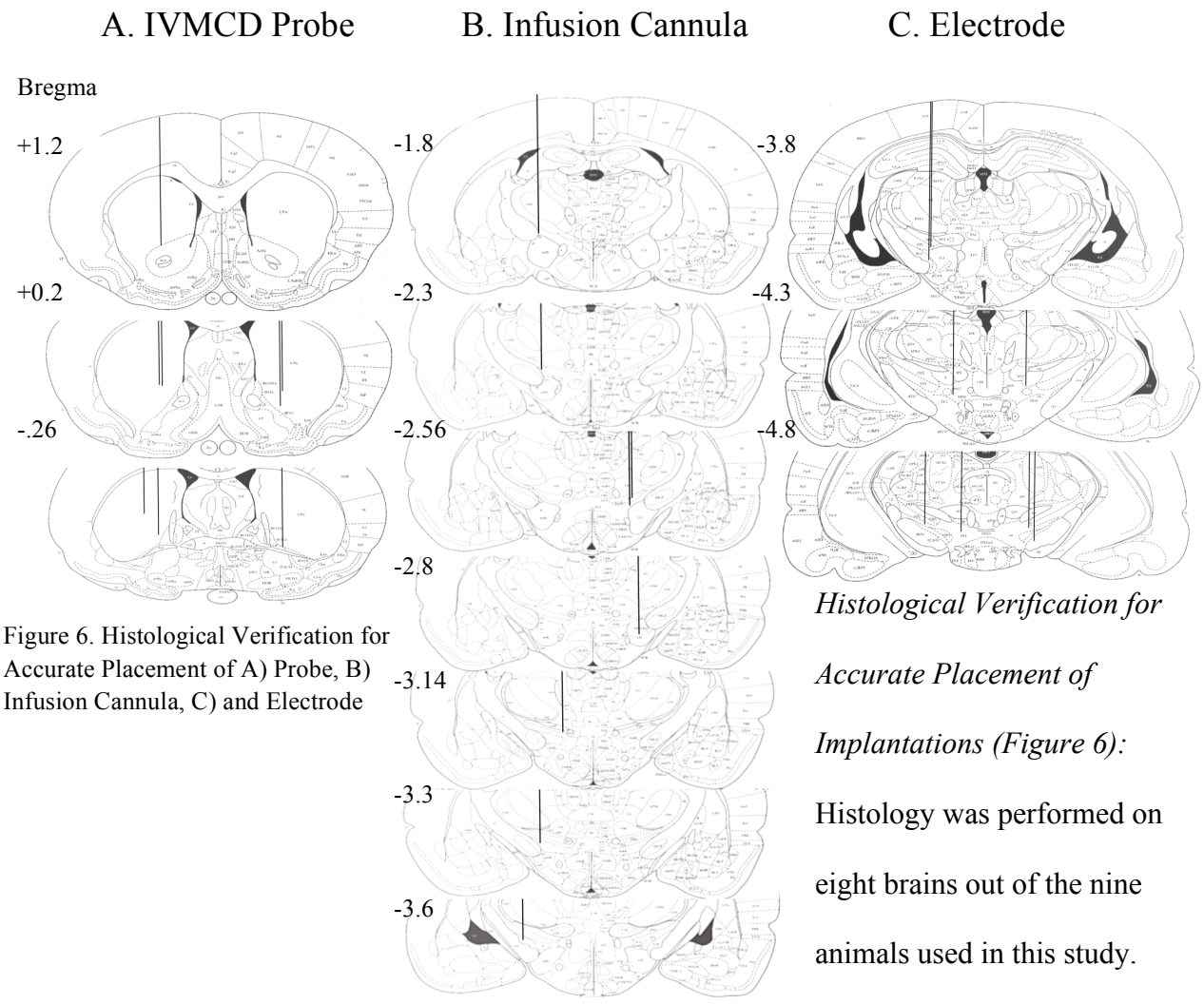

Figure 6. Histological Verification for Accurate Placement of A) Probe, B) Infusion Cannula, C) and Electrode

Histological Verification for Accurate Placement of Implantations (Figure 6): Histology was performed on eight brains out of the nine animals used in this study. Accurate placement of probes in the striatum (A), infusion cannulae in the MFB (B), and electrodes in the MFB (C) were verified in each animal (see methods).

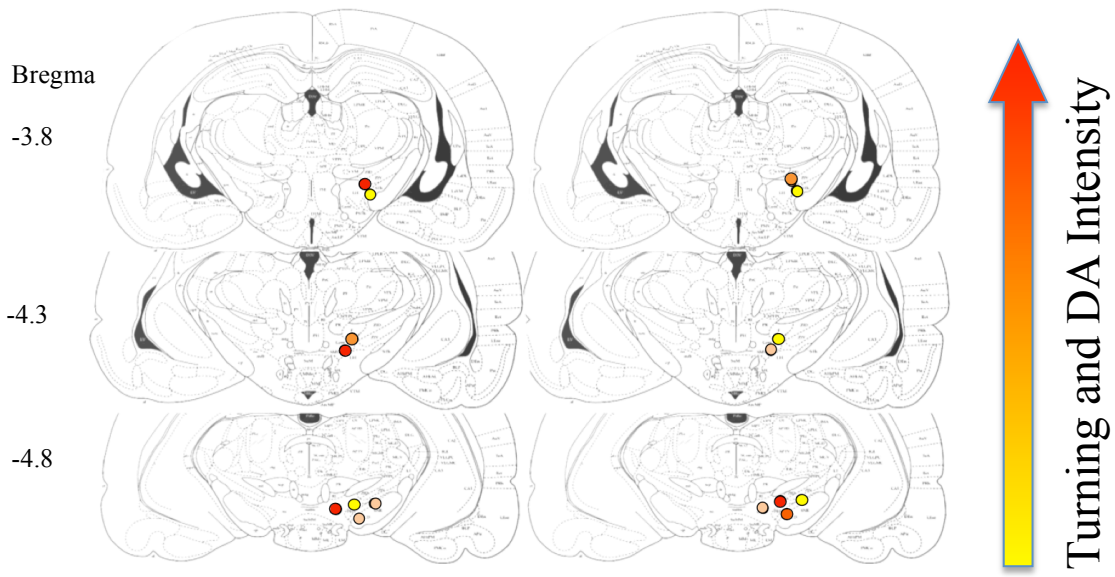

Figure 7. Anatomical Location of Electrode and Intensity of ES-Evoked A) Turning, and B) DA Overflow

Anatomical Location of Electrodes Associated with Intensity of Electrically Evoked Rotational Behavior and DA Overflow (Figure 7 A,B):

Coronal sections are displayed in an anterior to posterior direction from top to bottom, respectively. Intensities for both turning (A) and ES-evoked DA overflow (B) are indicated by a color spectrum where low intensities are associated to yellow and higher intensities are associated in a continuum to red. A) Intensity of turning is plotted for each animal, as a function of electrode location. Turning intensities ranged from 20-150 quarter turns within the 10-min ES time bin. It was casually observed that more medial placements generally evoked greater turning. B) Intensity of ES-evoked DA is plotted for each animal as a function of electrode location. Intensities of ES-evoked DA overflow ranged from 2-30 fmol/min during the 10-min ES time bin. The data do not show a clear relationship between ES-evoked DA overflow with electrode placement.

Discussion

Effect of Lidocaine on Electrically Stimulated DA Overflow and Rotational Behavior

The present data support the hypotheses that electrically induced DA release and ESRB are dependent upon Na^+ channels. ES applied at the nigrostriatal axons as they emerge from the substantia nigra evoked striatal DA overflow and ESRB. These neurochemical and behavioral responses were attenuated by the Na^+ channel blocker lidocaine microinfused downstream from ES at the MFB as it courses through the lateral hypothalamus. It is well accepted that ES produces depolarization to activate Na^+-dependent action potentials, subsequently producing exocytosis (Mulder et al., 1983). Therefore, the present data infer that ES in combination with IVMCD can be used to reliably evoke exocytotic-like DA release. The demonstration of Na^+ channel sensitivity complements additional unpublished data showing that ES-evoked DA overflow and ESRB are intensity- and frequency- dependent, as well as require Ca^{++} availability.

The premise of this thesis was to demonstrate that ES used in combination with IVMCD is a methodological approach that evokes exocytotic-like overflow of DA concomitantly with rotational behavior. Taken together, the present results combined with previously collected parametric data establish that electrically evoked DA overflow reflects activation of the physiological mechanisms involved in exocytosis. Specifically, ES mimics the depolarization of the membrane produced by an action potential, given that the sodium channel blocker attenuated both the neurochemical and behavioral responses to ES. ES, under the present conditions, evokes calcium-dependent overflow, given that calcium chelation and L-type calcium channel blockade reduced both DA overflow and ESRB. Notably, calcium-mediated vesicular docking is a characteristic of exocytosis. Finally, the evoked DA overflow and rotational behavior are

stimulus bound, frequency-dependent, and intensity-dependent, indicating that exocytotic mechanisms are recruited in these responses rather than being due to nonspecific effects (eg, damage-induced effects).

The significant contribution of the present thesis is that it provides validation for a methodology that produces a physiologically meaningful estimate of exocytotic events. Traditional techniques used to stimulate DA overflow have failed to produce physiologically relevant responses. Although amphetamine is widely used to produce DA overflow, it is not a desirable tool to understand changes in mechanisms of exocytosis because of its mechanism of action on the reuptake protein. In contrast, K^+ stimulation does increase extracellular DA by depolarization, but it does not effectively evoke a readily measurable behavioral response. Therefore, ES is an attractive tool used in combination with IVMCD.

The elegance of ES in combination with IVMCD is the ability to compare simultaneously evoked behavior with physiologically relevant responses. This study shows concomitantly evoked DA overflow and rotational behavior upon ES. Furthermore, both responses were significantly reduced by Na^+ channel blockade.Together these data validate the use of ES and IVMCD as an effective way to investigate presynaptic modulators of DA neurotransmission directly with changes in rotational behavior. It is important to note that the rate of ESRB was sustained across the five repeated trains of ES. These data suggest that DA levels evoked by ES using the present paradigm did not diminish throughout repeated stimulation, although this needs to be examined in future studies.

Effect of Lidocaine on ES-Evoked Metabolite Overflow

ES produced only a non-significant trend towards increases for extracellular DOPAC that was significantly augmented by lidocaine treatment. Extracellular levels of HVA were only significantly increased during the second 10-min sample following the last ES in comparison to the levels measured during ES. Similar to DOPAC, extracellular HVA was significantly augmented by lidocaine treatment. A similar pattern was observed for 5-HIAA. Therefore, the relationship between histological targets, DA, and ESRB was assessed, and this is discussed below (*Relationship between ESRB, DA Overflow and Anatomical Location of Electrode*).

In contrast to DA overflow, Na^+ channel blockade did not reduce the amount of metabolites after ES. Previous studies show a positive relationship between spontaneous levels of DA and associated metabolites (Castañeda et al., 1990). Surprisingly, lidocaine produced an increase in all three metabolites. Immediately following lidocaine infusion, three 10-min baselines samples were taken to assess the effects of lidocaine prior to ES. All three baselines for monoamine metabolites remained stable across the three samples. Given that, in the control condition, there was no significant change in extracellular DOPAC and 5-HIAA levels but there was an increase in HVA, a number of alternative explanations must be contended. First, it is possible that there is a delayed effect of lidocaine that manifests well after the 30-min test for the effects of lidocaine by itself. If this is true, then this study lacked a sufficient assessment of the effects of lidocaine alone on DA and metabolites without a confound of ES. This is a concern because another Na^+ channel blocker, halothane, is associated with increases in DA metabolism (Adachi et al., 2005). Second, there could be an interaction effect between lidocaine and ES that was observed in the enhancement of all three metabolites during and after ES in comparison to

control conditions. Further research will be needed to address this issue to elucidate the mechanism of action of lidocaine on changes in metabolism assessed with IVMCD.

Relationship between ESRB, DA Overflow and Anatomical Location of Electrode

An attempt was made to assess whether the anatomical location of electrodes corresponded to the effectiveness of ES to evoke DA overflow and ESRB. There was a minor pattern for more medially placed electrodes to evoke greater ESRB, but there was no readily observable relationship for electrode placement and ES-evoked DA overflow. In addition, there was no correlation observed between ES-evoked DA overflow and ESRB. These latter comparisons were actually beyond the scope of the present study, and a lack of any correlation is likely due to a number of factors: 1) A greater sample size was needed. 2) The intensity of ES was not allowed to vary randomly to create a true correlation with ESRB; in fact, a ceiling effect may have existed because of the high intensity of current used. 3) Electrode placements were narrowly clustered, limiting any variance in DA levels and ESRB as a function of randomized electrode placement. Properly designed experiments to investigate the relationship between anatomical placement of electrodes, ES-evoked DA overflow and ESRB are needed. A more succinct understanding of these relationships may improve the interpretation of hypothesis-driven research utilizing ES with IVMCD.

Future use of ES in Combination with IVMCD

ES in combination with IVMCD will be a valuable tool for behavioral neuroscience research of plasticity in DA systems. It is likely that common mechanisms are shared across development of the Central Nervous System, learning and memory, recovery of function, and drug addiction. Utilizing the concept of "turnover" that describes the life cycle of DA (Cooper et

al., 2003, p.230-231) based on a stream of factors (ie, synthesis, vesicular storage, exocytosis, receptor activation, reuptake, enzymatic degradation) provides a framework of presynaptic targets that modulate plasticity of neurotransmission. For example, this technique will be used to investigate such presynaptic mechanisms of neural plasticity that augment DA release in a denervated state, modeling Parkinson's disease, where surviving neurons have been previously shown to normalize extracellular DA levels (Stachowiak et al., 1987; Castañeda et al., 1990) and the compensatory mechanisms remain unknown. In addition, repeated stimulant drug exposure in a model of substance abuse disorder leads to an enhancement of DA release in the presence of drug-paired stimuli (Duvauchelle et al., 2000). Unfortunately, the presynaptic mechanisms that modulate conditioned DA release are largely unexplored. Until this is solved, measures of DA release will remain phenomenological. This innovative combination of ES with IVMCD will help to find key targets that may accelerate the development of therapies in DA-associated disorders.

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Curriculum Vitae

Alice Hernandez graduated with a bachelors of science in Psychology from the University of Texas at El Paso (UTEP) in 2012. She became interested in pursuing a career in neuroscience in 2010 when she began conducting research in Dr. Edward Castañeda's laboratory whose interests are in the neuroplasticity associated with Parkinson's disease and Substance Abuse Disorder. His laboratory combines, biochemistry, neuroanatomy and behavior to study compensatory mechanisms of Parkinson's disease and sensitization in Drug addiction. Alice received funding and awards to present her work at scholarly conferences. Alice is affiliated with the neuroscience community by virtue of her membership in the Society for Neuroscience, the National Hispanic Science Network, and as a Neuroscience Scholars Associate. She is also a scholarly member of several training and diversity programs including Summer Program In Neuroscience Ethics and Survival, Interdisciplinary Research Training Institute, Vulnerability Issues in Drug Addiction, and the National Hispanic Science Network. While pursuing her M.A. in 2013 she received the Bridge to Doctorate Fellowship (LSAMP) funded by the National Science Foundation. She received her M.A. in Experimental Psychology in 2015 and is now entering the Pathobiology program at UTEP where she is working toward obtaining a PhD.