The Placebo Effect in a Dish: Implications for Underlying Subcellular Mechanisms

A thesis submitted by

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In partial fulfillment of the requirements for the degree of

Master of Science

In

Pharmacology and Drug Development

Tufts University

Sackler School of Graduate Biomedical Sciences

May 2018

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Abstract

The placebo effect is well known as one of the founding premises for FDA-sanctioned clinical trials of new medications, yet there is very little scientific understanding of the cellular substrates of the phenomenon and lack of predictive biomarkers for the strength of the placebo response. Few prior correlative studies on the neurobiology of the placebo effect suggest the activation of central dopamine neurotransmission during a placebo response. The present project intends to further explore the cellular and molecular mechanisms that are linked to the placebo effect in peripheral paracrine and central monoamine-secreting cells. We follow an innovative and unconventional approach in trying to induce neurotransmitter release as a response to an inert stimulus in primary dissociated cells in culture.

At this first stage of our studies, we chose to conduct our experiments in primary dissociated rat adrenal chromaffin cells which are considered an excellent model of exocytosis for catecholamine-releasing neurons and at the same time are crucially involved at the systems level in setting the sympathetic tone and in disorders like the Cushing syndrome and hyperprolactinemia. We use the cutting-edge method of carbon fiber amperometry to assess catecholamine quantal release in real time in response to a high potassium stimulating solution in physiological temperature (37^{o}C). Following two stimulations within 10 minutes, we expose each chromaffin cell to only a physiological temperature saline and assess release of catecholamine quanta by cells based on the prior association between the active secretagogue and physiological temperature. Cells are otherwise bathing in room temperature aCSF media. We predict that the cells predisposed to a placebo response will release catecholamine quanta when exposed to physiological

temperature saline without the active secretagogue. We then proceed to identify differences in catecholamine kinetics and vesicular stores involved in the release of quanta from placebo prone cells versus non-responding cells.

The above study represents the first effort to describe the molecular signature of the placebo effect in an isolated cellular model. The end overall objective of such an effort would be the development of a screening assay for human patients identifying biomarkers for the potential of a placebo therapy in advance.

Acknowledgements

The completion of this study would not be possible without the support and guidance of my thesis advisor, Emmanuel Pothos, and the support of my lab mates Amber Xue, Rana Alabdali, and Aliya Butabayeva of whom I am incredibly thankful. Also, a sincere thanks to Jerrica Breindel, Alexei Degterev's lab (including Bing, Kerem and Suraj), the administrative staff at Tufts for their additional support.

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List of Abbreviations

aCSF	artificial cerebral spinal fluid
C	control cells (all stimulated)
CS	control cell stimulation
DRP	delayed release vesicle pool
E1	recording electrode
E2	stimulating or placebo electrode
ISI	interspike interval
KCl	potassium chloride
L	events with a left foot
MR	molecular release
PR	cells with placebo response
PRS	placebo response cell stimulation
RRP	rapid release vesicle pool
W	events without a left foot
WPR	cells without placebo response

Chapter 1: Introduction

1.1. What is a placebo?

Robert Hooper was one of the earliest to describe the placebo as "an epithet given any medicine adopted more to please than to benefit the patient" in his Medical Dictionary of 1811 (Clark & Leaverton 1994, pp. 20). However, this denotes placebo as used clinically, and not in a laboratory setting. Clark and Leaverton (1994, pp.20) utilize psychiatrist Arthur K. Shapiro's definition if a placebo as "any therapeutic procedure (or component of any therapeutic procedure) which is given deliberately to have an effect, or unknowingly has an effect on a patient, symptom, syndrome, or disease, but is objectively without specific activity for the condition being treated." They further extrapolate this definition to encompass "the use of placebos as alternatives to active treatment controls in randomized clinical trials".

1.2. Why is Placebo important?

Louis Lasagna, researched and then pioneered the use of the placebo effect with controlled clinical trials during asthma clinical tests. His "testimony at US Congressional hearings led to the Food and Drug Administration to demand proof of efficacy as well as safety of new drugs" by "promoting randomized controlled clinical trials as the gold standard". Dr. Lasagna's team brought the United States to rank as the first country "to have an efficacy standard in law (Tanne 2003, pp. 565)". By using the placebo as a control and baseline for comparison, the active treatment can be determined as effective if beyond this baseline. (Clark & Leaverton 1994, pp. 22).

1.3. How is placebo utilized in clinical drug development trials?

During clinical trials, the placebo is one of the controls on which to base the efficacy of a drug. If the drug does not exceed the efficacy of the placebo by … significance then it will not pass on to become a drug sold to the public. These trials are often done as blinded or double blinded experiments where different groups are administered either the placebo or one of the tested medicines without notice of which one they are given. Results most often are calculated at the end. Often times even the scientists are blinded so as not to bias the results.

1.4. What is the mechanism behind the placebo? What is known about the underlying neurobiology?

"Approximately 35 percent of subjects show satisfactory improvement on placebo, and placebo adverse reactions occur at approximately that same rate." These improvements or reactions can be measured physiologically or psychologically and "secondary to fluctuations in the course of illness with the passage of time, the effect of clinician-patient interaction, expectations of drug effect by both investigator and subject, or other aspects of the care environment" (Clark & Leaverton 1994). The mechanisms behind the placebo effect have two popular insights: classical conditioning and mental imagery. Classical conditioning is "effect of placebo administration on neurohormones (Clark & Leaverton, 1994)" and the primary interest of this paper.

Prior research which could be viewed today as relevant to an analysis of the placebo effect at a cellular and molecular level are classical conditioning studies with serotonin and *Aplysia* (Carew et al. 1981) as well as several more recent studies claiming

expectancy can influence the placebo effect (Colagiuri et al, 2015). The placebo effect for pain is open to conscious inhibition, but those governing hormonal responses are unaffected (Benedetti et al, 2003b as cited in Colagiuri et al, 2015). Furthermore, partial reinforcement (versus continuous reinforcement of placebo treatment) can cause a weaker response and more resistance to extinction of the ailment treated (Yeung et al. as cited in Colagiuri et al, 2015). While on a whole systems level, such findings support the need for more mechanistic studies at a cellular level to explore the molecular signature of the placebo effect.

From a neural standpoint, significant research done on pain analgesia and Alzheimer's disease implicates several brain regions with reduced activity under placebo treatment compared to active drug treatment, as shown in fMRI scans. These include the thalamus, primary and secondary somatosensory cortex, anterior cingulate cortex, insula, amygdala, basal ganglia, and right lateral prefrontal cortex. However, these response areas fluctuate between studies with only the anterior cingulate cortex and insula as constant. However, these two areas have also shown higher fMRI activity during some placebo analgesia studies and they coincide as activated areas during nocebo studies. Most importantly, the dorsolateral prefrontal cortex is strongly indicated to be crucial for both placebo and nocebo effects in both pain analgesia and Alzheimer's studies (Colagiuri et al 2015).

Other more specific research in gender and genetic differences is also of note. In gender studies, "the nonselective vasopressin agonist for both Avp1a and Avp1b receptors enhanced placebo effects in women but not in men" (Colloca et al., 2015 as cited in Colagiuri et al, 2015). Vasopressin is secreted in the adrenal medulla. Also, the

gene variant and exergonic SNP in the COMT gene, rs4680, seems to be involved in the placebo effect (Colagiuri et al., 2015). Of particular importance to our study, the COMT gene is linked to dopaminergic, norepinephrinergic, and serotonergic pathways since it is part of catecholamine metabolism. Other genes implicated in disease-specific manifestations of the placebo effect include MAO-A (depression), NR3C1(depression), DRD3 (schizophrenia), DBH (alcoholism), OPRM1 (mood response), FAAH (analgesia and affective state), SLC6A4 (depression), HTR2A (depression), TPH2 (stress), and 5-HTTLPR (stress) (Colagiuri 2015, 185).

1.5. What makes our methodology novel?

Experimental work on the placebo effect is almost exclusively conducted at a systems level with fMRI, whole animal studies and human patients. The present work is part of our efforts to understand any subcellular mechanisms associated with the strength of the placebo response in isolated primary catecholamine cells that share the catecholamine exocytosis apparatus with neurons, namely the adrenal chromaffin cells (see Figure 1.1). The gap in the molecular understanding of the placebo effect at the cellular level has been identified before (Price, 2013), but very little has been done so far.

A variety of techniques including cyclic voltammetry, amperometric testing, and whole cell physiological cryofixation have been used to understand the mechanisms of neurotransmitter release in adrenal chromaffin cells. Likewise, these techniques have been paired with bathing the cells in different solutions, time allowance to gauge re-uptake, and stimulating with different solutions.

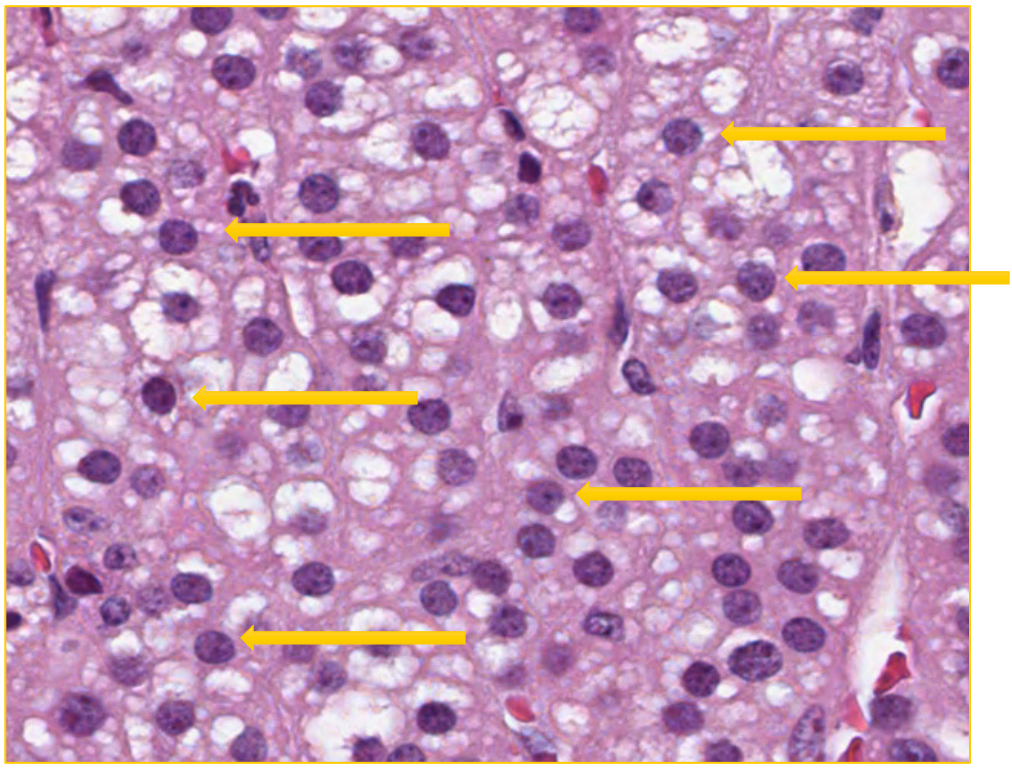

Figure 1.1: Chromaffin Cells (dark stain), located inside the Adrenal Medulla. Yellow arrows point out specific cells. (Adapted with permission from The Human Protein Atlas (Uhlen, M. et al. 2005). (Changes were made by cropping, adding yellow arrows, and a yellow border.)

In order to see if chromaffin cells display a placebo effect, we attempted to pair an active secretagogue stimulation (unconditioned stimulus) with physiological temperature (conditioned stimulus) and eventually tested to discern whether physiological temperature saline alone could elicit a response. At first the cells were bathed in a buffer of artificial cerebrospinal fluid (aCSF) at room temperature and stimulated (puffed) twice with a physiological temperature high K^+-high Ca^{2+} stimulating solution for 6 sec at five minute intervals followed by a third physiological temperature aCSF puff after another five minutes. Five minutes intervals were employed to allow the cells time to recuperate from stimulation.

Cells which secrete neurotransmitters are highly modular, sensitive to subtle environmental changes such as extreme changes in temperature through transient receptor potential family cation channels. Nociceptive neurons are good example of this as cold temperature activates ANKTM1. Since chromaffin cells transmit neurotransmitters and are highly similar in release apparatus to neurons it is believed they will have a similar sensitivity to temperature (Oppenheimer 2016, 5).

1.6. Why are chromaffin cells being used instead of neurons?

The adrenal gland is part of the endocrine system which secretes hormones and the adrenal medulla is a principal site of conversion and release for catecholamine neurotransmitters. Catecholamine are released upon chemical or hormonal excitation of the cell into the extracellular space in a quanta fashion (Oppenheimer 2016, 6). Thus, chromaffin cells which make up the adrenal medulla are a good model for neuronal exocytosis.

1.7. How will this enhance our understanding of the placebo effect?

The results will be analyzed by two different methods. As a first step, Axograph will be used to analyze the signals received through carbon fiber microelectrodes. Amplitude (height), half-life (t ½), full time duration, and location of each quantum of neurotransmitter released will be measured. From the measurement, the number of molecules released, and the inter-spike interval (ISI) value will be calculated. Further analysis will be made to verify if the vesicles are from rapid release pools (RRP) or delayed release pools (DRP). As a result, our studies may provide a unique insight on

what type of vesicles participate in neurotransmitter exocytosis induced by active secretagogues and drugs versus exocytosis induced by placebo stimulations.

Chapter 2: Methods and Materials

2.1. Characterization of Rodents Used

Animals used in these experiments were Sprague Dawley male rats ranging from 1 – 8 months that were housed in an inverse light cycle (lights off at 8:00AM and on at 8:00PM) in the Tufts Center for Neuroscience Research with no dietary restrictions. Although similar in proliferation to mice in cell culture (Tischler et al. 1997, 219), the rat adrenal gland is easier to locate for extraction.

2.2. Cell Culture Protocols and preparation steps

Following modified procedures from previously published protocols (Oppenheimer 2016, 7-8) and in accordance with the laboratory's IACUC approved animal protocol, at least 24 hours beforehand equipment was cleaned with ethanol (including all pipette tips, reusable pipettes, disposable pipettes, kimwipes, disposable petri dishes, and tubes), all solutions were made, cell culture dishes were washed with nano-pure water and ethanol, and all materials (except solutions) were left to air dry under UV light in the hood. Also, a water-jacketed incubator was set with 5% CO_2 levels and 37°C at least 24 hours in advance.

On the day of the cell culture, six aliquots of Locke's buffer were prepared using a pre-made sterilized solution under the hood to remain sterile (see Table 5.1. for protocol). Of the six aliquots, two were placed in the incubator (as well as the chromaffin cell medium (solution protocol in Table 5.2.) two in the fridge or bucket filled with ice and two at room temperature under the cell culture hood. Additionally, the enzyme was prepared in the dark (due to light reactivity). Composed of 10-11mg collagenase A and

18mg of bovine serum albumin, the enzymes were weighed and placed together in a sterilized container under the hood. Also placed under the hood were an electric heater/stirring platform spritzed with ethanol along with a water bath (beaker filled to 400 mL of nano-pure water), a Styrofoam holder, a thermometer, a flat bottomed lidded container which fits in the Styrofoam container with miniature stirring rod), and a microscope.

Lastly, laminin aliquots (formulated at 0.05 Tris/0.15 M NaCl and pH 7.4) were taken out of the -80°C and thawed. 50 mm cell culture dishes with glass wells were plated with 10 µl of the laminin solution. Dishes were then placed in a sterile tray either at 5°C (refrigerator) overnight or in the incubator at 37°C for at least two hours before plating chromaffin cells.

2.3. Chromaffin Cell Extraction

2.3.1. Euthanizing

Rats were injected intraperitoneally with a 1:1 ratio ketamine and xylazine cocktail dependent on their weight at 0.1ml/100g. Once unresponsive, rats were euthanized. The body is sprayed with ethanol and placed under the cell culture hood while observing sterile technique.

2.3.2. Adrenal Gland Removal

The main body cavity was opened through skin incisions right below the rib cage in a V-shaped center cut. The adrenal glands were identified and then removed by locating the kidney above the kidney. Once the adrenal gland was located, forceps were

used to extract it. Once removed, both adrenal glands were placed in an ice cold 6mL Locke's buffer.

2.3.3. Preparing for and Digesting the Adrenal Gland

The two adrenal glands were cleaned off and each gland was cut once through the center creating an end amount of four pieces. Using a disposable pipette, they were placed in a 6mL Locke's buffer with the two enzymes of 10-11mg Collagenase A and 18mg bovine serum albumin. The adrenal glands were digested in the water bath for 70 minutes at 37^{o}C. Every 10 minutes the contents were gently titrated using a disposable pipette.

2.3.4. Plating the Chromaffin Cells

Supernatant was moved using a disposable pipette into a sterilized 15 mL test tube. This test tube was centrifuged at 1,000 RPM for five minutes (or until clear/transparent solution was seen in the tube). Without disturbing the pellet, the supernatant was removed and disposed of. The pellet was then re-suspended within 6 mL of Locke's buffer from the incubator and gently titrated with the Pasteur pipette 10 times to wash the cells and wash out the enzymes. Again, the test tube was centrifuged at 1,000 RPM for five minutes (or until the contents were clear/transparent), the supernatant was slowly removed and discarded without disturbing the pellet and the pellet was re-suspended in 1 mL of incubated chromaffin cell culture medium. 100 µL of the cell suspension in the chromaffin cell medium was placed in the center of the lamininted glass well of each dish. Once the cells were plated, the dishes were placed in the incubator for

1.5 – 2 hours. Then, the glass wells were gently flooded with 2.5 – 3 mL of chromaffin cell culture medium. 24-96 hours post plating, the cells were ready for electrophysiological testing.

2.4. Electrophysiology Testing

2.4.1. Preparation and Hardware

Following protocol(s) similar to Oppenheimer 2016 (8 - 11, 15) with minor differences, an inverted microscope (Nikon Eclipse TE300) on an air table was used as well as two MP-285 Micromanipulators (one for each electrode; from Sutter instrument company), Picospritzer II (Parker Hannifin Corp.), Axopatch 200B Integrating Patch Clamp (Axon Instruments), and a MacBook Pro fit with AxoGraph software, an electrophysiology program. Aliquots of stimulating solution and aCSF were placed in a water bath at 37°C and cell cultures were sitting in aCSF at room temperature. We then employed carbon fiber amperometry which can

> "record cellular activity in real time and… [has a] high specificity for oxidizable molecules like catecholamine. Secretion is achieved by exposing the cell to depolarizing agents (referred to as secretagogues), such as K+, Ca^{2+}, or a mixture of both, from a stimulating electrode. This elicits a reaction in the cell analogous to *in vivo* neurotransmitter signaling (Oppenheimer 2016, 8-9)."

The cells release catecholamine in variable sizes of quanta. Stimulating electrodes and puffing electrodes were prepared according to Chapter 5 Appendices Section 5.2. where the puffing electrode containing the physiological temperature stimulating solution and the puffing electrode with the physiological temperature aCSF were kept separate to

avoid contamination. The recording electrode and puffing electrodes were placed at 40° angles under the microscope and in the microcontroller holders. The recording electrode was filled with 3M KCl and the puffing electrode was filled with assigned solution as per the protocol in Table 5.3. or Table 5.4.. The recording electrode was attached to an Axopatch 200B amplifier set at an oxidation potential of 700mV ground through a Ag/AgCl ground wire., Data was acquired through the AxoGraph X software on a MacBook. The puffing electrode was connected to a Picospritzer II pressure pulsar (Parker Instrumentation) which was concurrently connected to a Nitrogen tank (Airgas Inc.) to facilitate the puffing action.

2.4.2. Testing

Each cell culture dish was individually tested within four days after the incubation period of 24-96 hours. Approximately 10 cells were tested for each dish. Upon removal of the cell culture dish from the incubator the chromaffin medium was removed and replaced with 1 mL of room temperature aCSF. The recording electrode was gently placed on the surface of a viable cell. A viable chromaffin cell exhibits phase contrast with multiple organelles being visible at a 25X magnification. Likewise, the puffing electrode was placed approximately 15-18μm to the right of the cell.

For the first stimulation, the cell recording started at zero and then was stimulated after five seconds. Once a cell was stimulated, a period of five minutes was given to the cell to recuperate. Then the cell was stimulated again with the warm stimulating solution. If the cell again demonstrated stimulation, then another period of five minutes elapsed before administering a puff of warm aCSF. Release events after the third stimulation (of

the inert aCSF in physiological temperature) were considered a placebo response by the cell, making it feasible to work on a model of the placebo effect at a cellular and molecular level.

2.5. Data Analysis

After all the data was collected and saved using Axograph. Averages and statistical analysis are provided in Tables 5.5. – 5.12.. Kinetic parameters included the amplitude (height; in pA), width (in msec), the time location, the half-life (t1/2 or width at half-height), area under the curve, and the time length of each event. Overlapping events were discarded because according to Calvo-Gallardo, et al. (2015, C11) "multiple spike events (overlapping spikes) indicate near-simultaneous quantal catecholamine release from vesicles belonging to a RRP [Rapid Release Pool]". This claim is based on previous research they conducted with spontaneously hypertensive rats and normotensive rats (Calvo-Gallardo et al 2015 referred Miranda-Ferreira et all. 2008). Criteria for these event were based primarily off of Calvo-Gallardo et al (2015), but also supported by similar findings in Table 2.1

All cells were stimulated three times at five-minute intervals. Each set of stimulations was then averaged per cell in all parameters. The measurements which were additionally calculated included the average height of an event per cell, the average width of an event per cell, the average interspike interval (ISI) value per cell, and the number of molecules released on average per cell. Examples of such measurements are in Figure 2.1. I(max) is the height, t1/2 is the half width, the shaded grey is the area under the curve (AUC), the distance from the start of the peak on the x-axis to the end of peak on x-axis

is the width of the event, and the location is the center of width. The ISI value is taken as the distance between the center of each event and then averaged. The higher the ISI, value, the lower the frequency of quanta release (Calvo-Gallardo et al. 2015; Viskontas 2007).

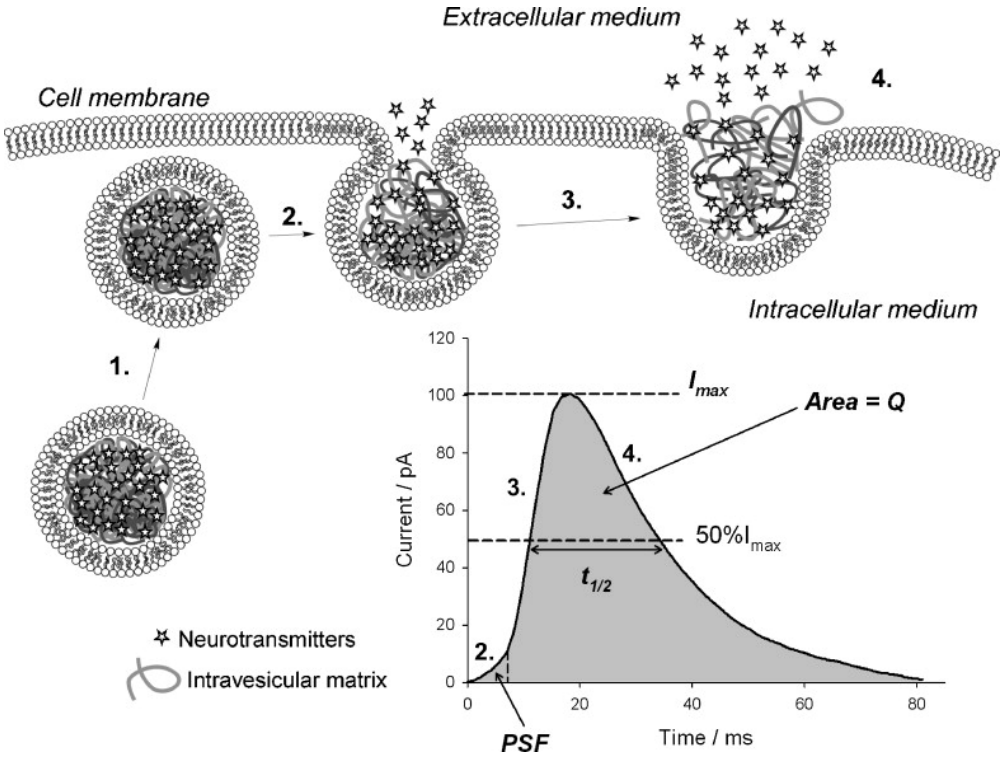

Figure 2.1: Schematic of Exocytosis. Vesicles in the cytosol approach the cellular membrane to release its contents through exocytosis. At stage 3, the vesicle has a Snare Complex latching to the membrane enabling the contents of the vesicle to be released into the extracellular space as in 4. The Snare Complex is what latches onto the cell membrane is denoted by a 'foot' at base of an event similar to the PSF in the graphical image below the image of exocytosis. However, the foot when Snare Complex is present is believed to be larger than what is represented as the PSF (Reprinted with permission from Elsevier and Copyright Clearance Center. (Meunier, A. et al. 2014)).

The width is used along with Faraday's equation to find the number of molecules released during an event: Q = nNF where

"Q is the charge integrated under the amperometric peak, n is the number of electrons transferred per molecule of analyte oxidized (2e- for catecholamine) and F is the Faraday constant (96,485 C/mol) (Mellander et al. 2012)."

The amplitude or height indicates how much neurotransmitter release takes place during exocytosis. The width of the event indicates how long the cells take to complete the process of exocytosis.

Table 2.1: Characterization of Chromaffin Cell Events

Appearance	Indication	Source
Overlapping events	RRP	Calvo-Gallardo, et al. 2015, C11
Singular events without foot/feet	RRP	Calvo-Gallardo, et al. 2015, C11
Foot without event	RRP	Albillos 1997, 511
Singular event with foot/feet	DRP	Ales et al. 1999, 44; Calvo-Gallardo et al 2015, C17; Mellander 2012, 907

The control cells indicate the cells were only given the stimulating solution every five minutes where the 1st stimulation (C1) is followed after 5 minutes by the 2nd stimulation (C2) followed after five minutes by the 3rd stimulation (C3). The placebo responding cells (PR) are grouped by their first, second, and third stimulation (PR1, PR2, and PR3), which are also separated by five-minute intervals and the last stimulation is the

physiological temperature aCSF buffer instead of the physiological temperature stimulant.

2.5.1. Event Feet

To further extrapolate on the release mechanisms of the chromaffin cells, the kinetic calculations further involved the category of those events with a left foot. All data in which events displayed a left foot as indicated in Table 2.1, were isolated from the overall data in both control and placebo responding cells to understand the mechanism in which cells release catecholamine. Thus, the left foot is an indication of fusion pore flickering during exocytosis that may be associated with a specific pool of vesicles (Table 2.1).

It may be assumed here based on the research of Albillos (1997), Ales, E. et al. (1999), Calvo-Gallardo et al. (2015), and Mellander (2012) that the major defining difference between a RRP and a Delayed Release Pool (DRP) is a transient fusion pore opening (see Figure 2.1). In the literature, the fusion pore is sometimes represented on its own as a smaller event or as a transient opening leading to full fusion of the vesicle to the plasma membrane which looks like a foot attached to an event. In Albillos (1997) these transient events are called 'kiss and run' events, because they do not release everything as the membrane does not fully fuse. Ales et al. (1999) believe this could also be a rapid release mechanism until a "phase of low electroactivity exists (44)" to take care of cell maintenance and utilize DRP. Calvo-Gallardo et al (2015) appears to support this idea during discussion of 'flickers' denoting the transient event, which leads to a much larger event and release of catecholamine during exocytosis.

Furthermore, Albillos (1997), does support the notion that the contents of the event with and without the foot remain primarily the same, further supporting the idea of the foot as part of the secretory response and not as an extremely small event. Mellander (2012), does dispute the variance in amount released, but in a much more in-depth review of a pre- versus a post- foot. Therefore, since the events, which involve docking to the membrane require more time and stimulation to release their contents, these are DRP. Whereas, the events which rapidly rise and fall in a typical event without a 'foot' are more like the RRPs described in Calvo-Gallardo's (2015) paper and so are deemed RRPs. Significant presence of the DRP in placebo responding cells would likely indicate that a cellular placebo response require time for the SNARE complex to attach to the cell membrane and release the quanta. A schematic of a pre- or left foot indicating attachment of the Snare Complex is present in Figure 2.2.

Furthermore, previous research by Mellander et al (2012) characterizes events with 'pre spike feet' to have significantly different kinetic measurement variations than normal events without feet. While Mellander et al (2012) describes the feet in categories of pre-, pre/post, and post, the pre- and pre/post groups are combined in this analysis. Likewise, the number of SNARE complexes is believed to be lower with lower exocytosis rate, lower foot charge, and lower foot time for pre/post feet. The post feet are ignored because they poorly indicate the relationship of DRP and RRP to the events as is important for understanding the placebo effect at a molecular level. In fact, when only pre-foot is present, it is believed eventual full distension of the vesicle with complete release of the contents into the cell membrane occurs which is indicated by slower events indicated by large width with their significantly higher amplitudes. Thus, if cells are more

prone to full fusion and considered DRP then left footed events will appear with higher amplitudes and larger widths (Mellander et al. 2012).

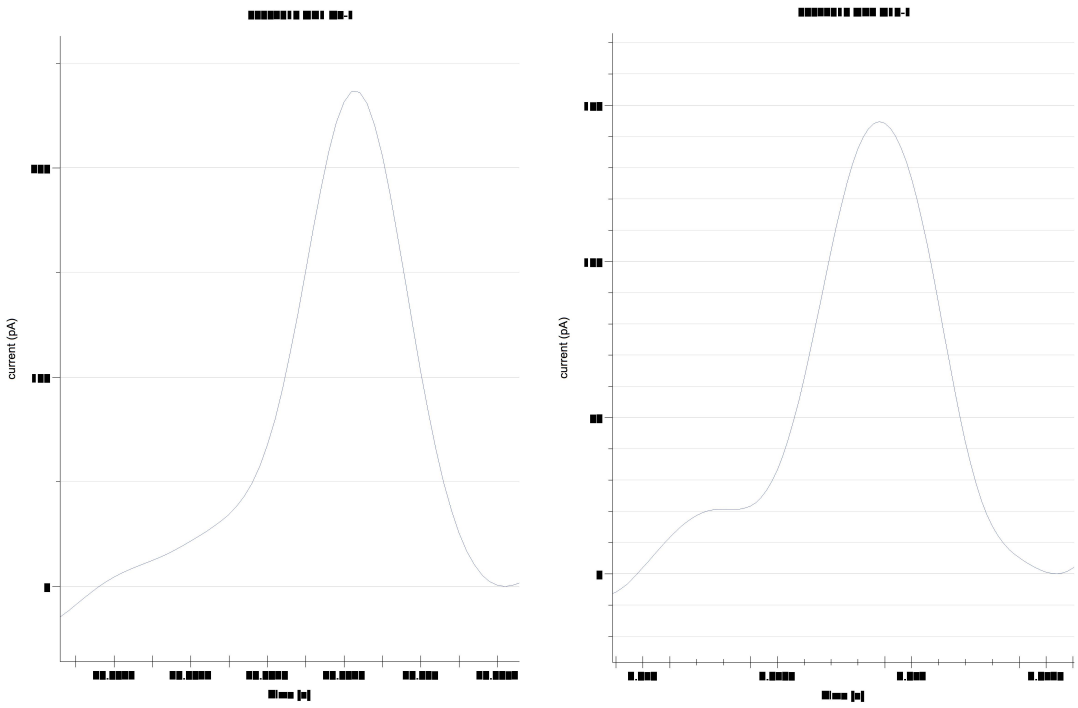

Figure 2.2: Examples of Left Feet. Taken from events during this experiment, these images demonstrate a classical event such as in Figure 2.1, but with also a left foot. The image on the left is a ramp left foot and the image on the right is bump left foot, but both are left feet.

2.5.2 Statistical Analysis

Statistical analysis was done using Prism Software. Statistical significance was determined between C and PR cells by using unpaired t-test, two-tailed for P values < 0.05 using Welch's Correction. If statistical significance was established, 1-way ANOVA with repeated measures was used with the Wilcoxon test to assess all cells retested at different time points ($p < 0.05$ denotes significance).

Chapter 3: Results

Six rats were euthanized for the results of this thesis. For each of these six rats, about 10 cell culture dishes were made bringing the total number of cell culture dishes to approximately 60. 10 of these dishes were used for the control procedure leaving 50 dishes for detecting a possible placebo response. An average of 10 cells with a first positive stimulation was in place. Of the cells, which were recorded, the number of cells which had a placebo response, those which did not have a placebo response, and the number of cells which acted as a control are recorded in Table 1.

Table 3.1: The Number of Cells Which Did or Did Not Responded to Placebo and the Control

	With Placebo Response (PR)	Without Placebo Response (WPR)	Control (C)
# of cells	152	30	60

The averages of the kinetic parameters are in Tables 5.5. 5.12.. Figures 3.2 – 3.6 represent a summarization of all of the PR and C data in comparison with one another. Values are arranged by stimulation group (i.e. C1, C2, C3, PR1, PR2, PR3) with a 'L' denoting if the values only contained events which have a left foot. Table 3.2 examines in more detail the percentage differences of events overall versus L. Figure 3.1 presents this data in a bar graph.

Table 3.2: Number of Cells Total versus Cells with a Left Foot

Total Number of Cells Stimulated	Number of Total Cells with a Left foot	Number of Cells with a left foot during S1	Number of Cells with a left foot during S2	Number of Cells with a left foot during S3	
152	137 (90.13% of total)	89 (64.96% of all PR with left)	82 (59.85% of all PR with left)	75 (54.74% of all PR with left)	PR
30	23 (76.67% of total)	15 (65.22% of all WPR with left)	14 (60.87% of all WPR with left)	0 (0% of all WPR with left)	WPR
60	55 (84.61% of all total)	30 (54.55% of all C with left)	38 (69.09% of all C with left)	31 (51.67% of all C with left)	C

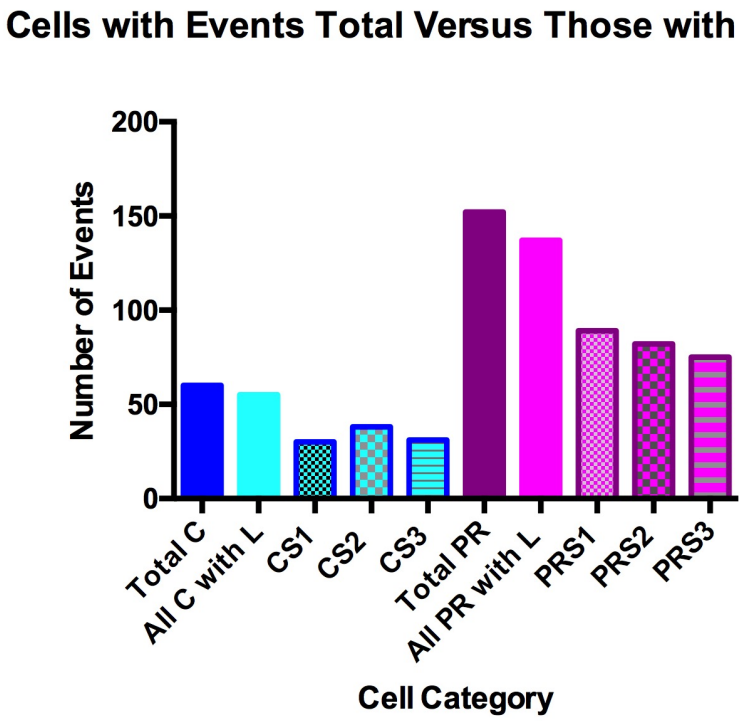

Figure 3.1: Cells with Events Total Versus Those with Left Foot. Bar Graph of Table 3.2 Representing the Total Number of Cells verses those with a Left Foot. Blue are the total control (C) cells, cyan are the total C cells with only L, patterned blue/cyan columns are the stimulation sequence of control cell stimulation (CS) cells with only L, purple are all the placebo responding (PR) cells, fuchsia are all the PR cells with only L, and patterned PRS columns are PR cells with only L events and are numbered in stimulation sequence. No statistical analysis was made because each column is a singular value.

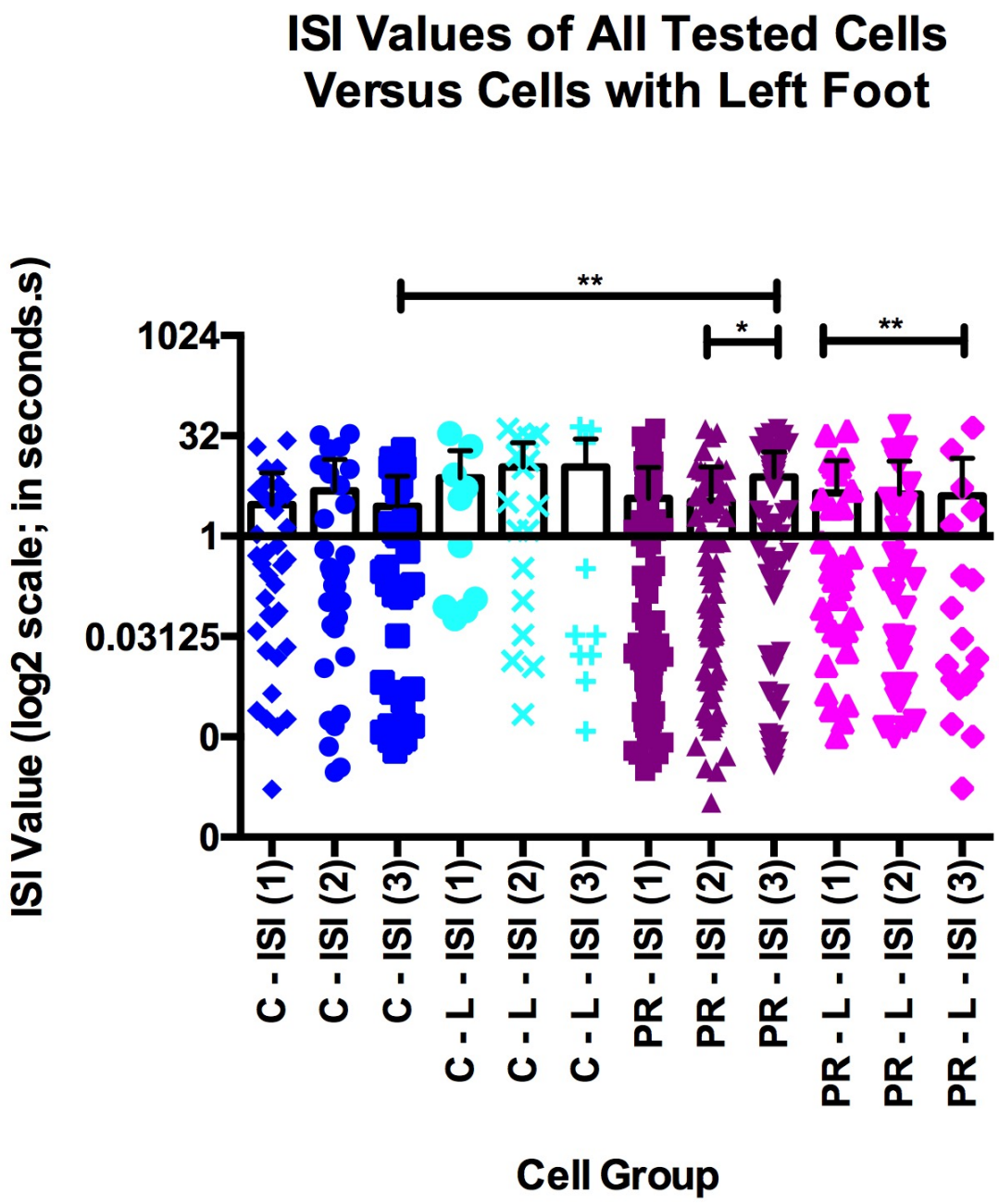

Figure 3.2: ISI Values of All Tested Cells Versus Cells with Left Foot. ISI Values for each Cell Group Type, Control (C) in their first through third stimulation set (1, 2, 3) and Placebo Response (PR) in its first and second stimulation response (1,2) and stimulation with placebo (3) are compared against those with only L (left foot). Columns present the mean data with bars indicating SD. Blue are the C cells, cyan are the C cells with only L, purple are the PR cells, and fuchsia are the PR cells with only L.* denotes $p < 0.05$ and ns = not significant. ISI = interspike interval. Scale is Log2.

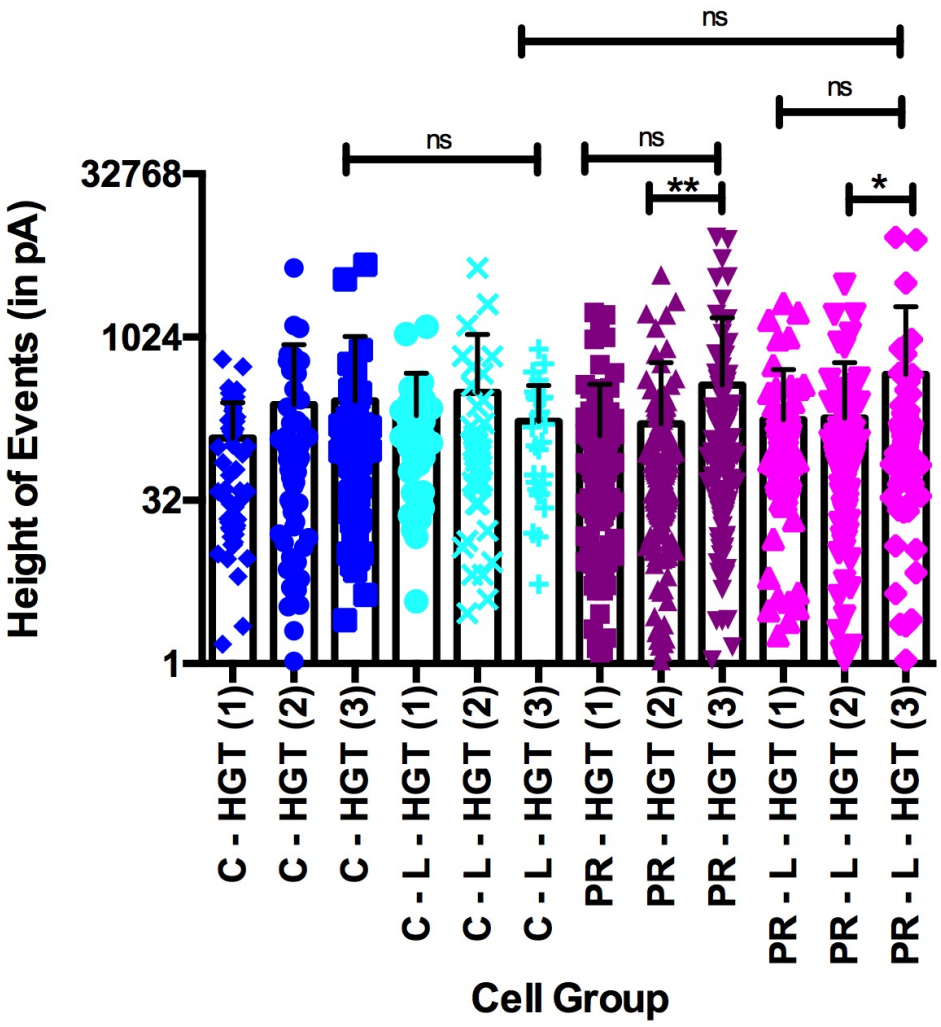

Figure 3.3: Height of All Tested Cells Versus Cells with Left Foot. The heights of the control (C) and placebo response (PR) cells are compared here amongst their stimulation set (1 = 1st, 2 = 2nd, and 3 = 3rd sequence stimulation where the 3rd for PR is a placebo). Individual cell averages are represented by scatter where blue are the C cells, cyan are the C cells with only a L, purple are the PR cells, and fuchsia are the PR cells with only a left foot (L). * denotes $p < 0.05$ and ns = not significant. CS = control cell stimulation; PRS = placebo responding stimulation; HGT = this is the height value. Scale is Log2.

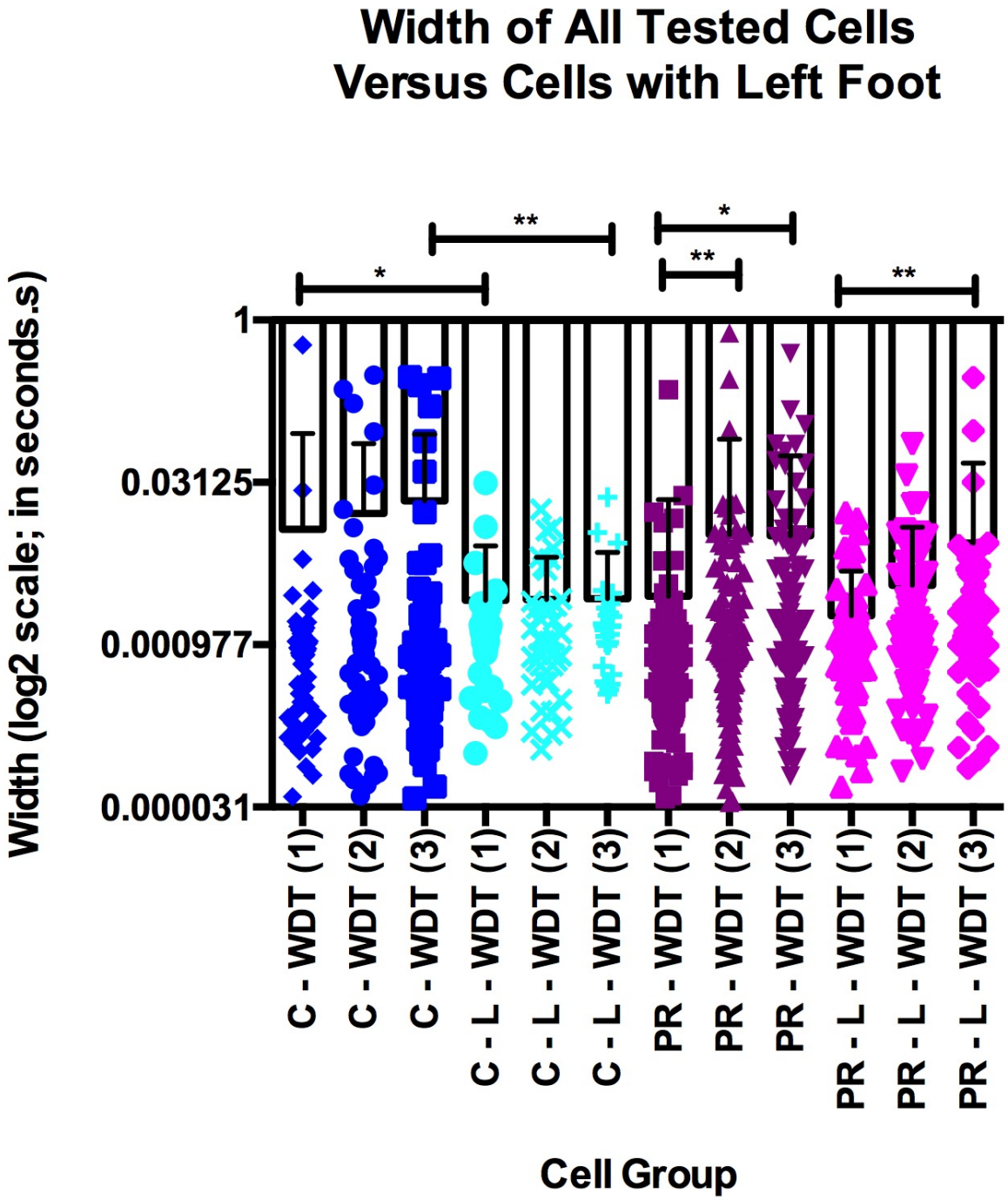

Figure 3.4: Width of All Tested Cells Versus Cells with Left Foot. Similar to Figures 3.2 and 3.3, singular data with columns at the mean and bars representing SD. Control cells (C) 1st, 2nd, and 3rd stimulation in sequence (1, 2, 3) are compared to placebo responding cells (PR) stimulations sequence (except 3 = placebo) and the data of each category containing L information. Blue are the C cells, cyan are the C cells with only L, purple are the PR cells, and fuchsia are the PR cells with only L. To see individual cell values more clearly, scale is log2.

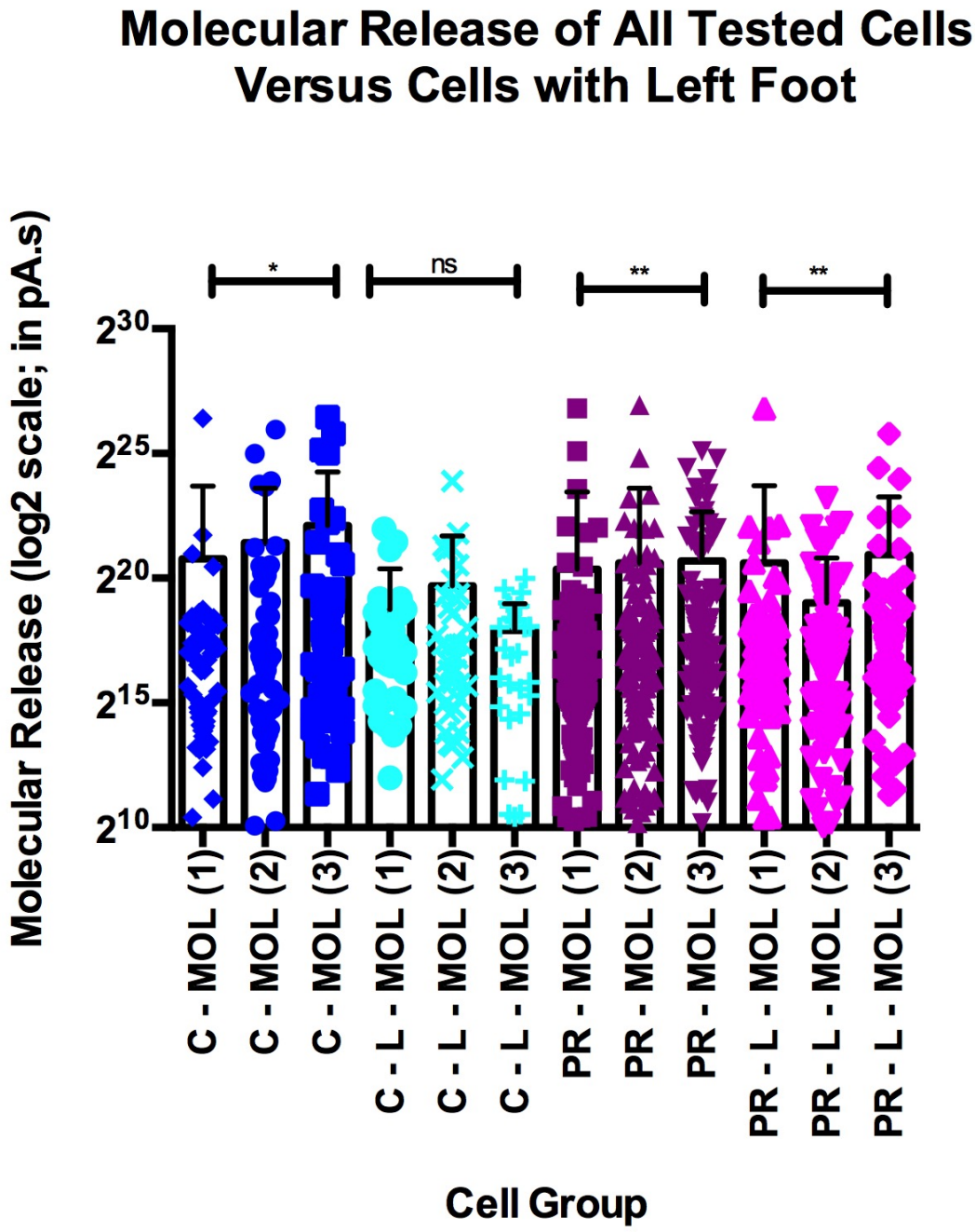

Figure 3.5: Molecular Release of All Tested Cells Versus Cells with Left Foot. The molecular release from each cell during exocytosis is presented here as individual data with bar graph reaching the mean value for each category and error bars. 'C' indicates control, 'PR' placebo responding, 'L' when only events with left feet are representing. 1, 2, and 3 are the stimulation set where 3 is the placebo for the PR group. Scale is log2.

Chapter 4: Discussion

The present study constitutes a preliminary attempt to establish a placebo effect model at a cellular and molecular level. The kinetic measurements indicate the PR cells compared to the PR L only cells have a greater propensity to release DRP vesicles during exocytosis than the C cells. In general, over 50% of the cells in any sequence of stimulation of either the PR or C cells had left feet with a total of over 75% of the overall PR or C cells exhibiting at least once a L during an event (see Table 3.2 and Figure 3.1). The kinetic parameters for these events were averaged broadly by the sequence in which the C or PR cell was stimulated and then partitioned to examine only cells which exhibited a L. As a reminder, ISI, width, amplitude, and molecular release were collected and placed in Figures 3.2 – 3.5.

In Figure 3.2, the ISI values only showed significant changes between the third overall C stimulation and the 3rd stimulation of all the PR cells, between the second and third stimulation of all of the PR cells, and between the first and third stimulation of only the L PR cells. The difference between the third stimulation of all PR cells to the second stimulation of the same PR cells and to the third stimulation of the C cells appears to be because the third stimulation of the PR cells show less variance and more accumulation of larger ISI values. This further indicates as suggested in the discussion of the methodology that all the PR cells during the placebo administration have a slower response rate of events. However, this does not appear to be attributed to the cells with a left foot because they show significantly more variability with increased lower ISI values when compared to the first stimulation of only L PR cells.

On the other hand, the height difference in Figure 3.3 between the second stimulation of all the PR cells and only L PR cells to the third stimulation of both all PR cells and only L PR cells is significantly different. This significance is attributed to an increase in high amplitude values for the third placebo stimulation. This correlation indicates that the height increase can be attributed to the L PR cells within the all PR cells group. Likewise, this correlates with Mellander et al. (2012) discussion of L cells containing greater amplitude heights.

Further support of Mellander et al. (2012) supposition of L events is the significantly increased width between the first all C group and first L C group and the third C group with the third L C group (see Figure 3.4). While it would be thought the L C groups would have a significant difference in height too, they only show this significant difference in width where the widths are larger and more variant for overall 1st and 3rd stimulation of C cells than the L only C cells in first and third stimulation. On the other hand, while no significance exists between the control group and the placebo tested group, a gradual increase in width is apparent at a significant level both in overall PR cells and within the L PR only groups. This may indicate that the placebo responding cells are more likely than the C cells to transition to full vesicle fusion to the membrane when administered a placebo stimulation. However, this is not to say the C cells do not exhibit full vesicle fusion at all.

Coincidently, within all PR, only L PR, and all C have a significant increase in MR values occur between the first and third stimulation. However, this is surprisingly not the case for the only L C cells. This indicates, the cells receiving a placebo stimulus as the third stimulation may have increased DRP than C cells especially since the only L PR

cells have this increase in MR values to coincide with the all PR cells. This coincides with the discussion of DRP and only L events as discussed in the methodology when referring to Mellander et al. (2012).

The kinetic measurements of the placebo responding cells appear to correspond to the discussion in the methodology of what parameters only L cells normally have and what makes them good candidates for releasing DRP. This parameters were an increase of amplitude, an increase in width, and an increase in MR. Although not necessary, the decrease in ISI value for L only PR cells indicates they exhibited increased fusion flickering.

Overall, the results, albeit preliminary, indicate the propensity for the PR cells to undergo DRP exocytosis which may be a useful starting hypothesis for identifying the placebo effect at a cellular/subcellular level. Eventually, it will be interesting to isolate the cells singularly, performing a plaque assay during stimulation to acquire the exact contents of release for comparing the control to the placebo responding cells as this will further extrapolate on any "modulation of exocytosic events by compounds coreleased with catecholamines" (Arroyo et al. 2006) which can slow down the release process; as well as single cell PCR to identify changes in mRNA in different regulators of catecholamine exocytosis. Further supporting experimental work could include the Fura-2 assay to view the exact calcium movement in and around the cell(s). Otherwise, this is a potentially important indication of the difference in rapid release and delayed release storage vesicles involved in the placebo effect that can be
further built on through complementary tools.

Chapter 5: Appendices

5.1 Reagents

Table 5.1. Locke's Buffer (for 1L)

Reagent	**Molecular Weight (g/mol)**	**Amount**
NaCl	58.44	8.99976 g
KCL	74.55	0.26838 g
NaHCO3	84.01	0.470456 g
Glucose	180.2	1.00912 g
HEPES	238.3	2.383 g

Table 5.2. Chromaffin Cell Medium (for 250 mL)

Reagent	**Amount**
Fetal Bovine Serum	25 mL
DMEM (Cellgro, Mediatech Inc. # 10-013-cv; with high glucose L-glutamine and sodium pyruvate)	223.75 mL
PNC (Penicillin, 50 unit/mL) + Streptomycin (50 µg/L); (antibiotics, dilute 1:200 from stock)	1.25 mL

Table 5.3. Stimulating Solution (80 mM KCl/6.0 mM CaCl2 Saline with PO4) (for 1L)

Reagent	**Concentration**	**Amount**
NaCl	67.2 mM	3.647 g
KCl	80.0 mM	5.964 g
NaH_2PO_4	1.0 mM	0.120 g
Hepes	10 mM	2.384 g
CaCl2	1.2 mM	0.666 g
MgCl2	1.0 mM	0.204 g
Glucose	25 mM	4.506 g

Table 5.4. aCSF (for 1

Reagent	**Concentration**	**Molecular Weight (g/mol)**	**Amount**
NaCl	124 mM	58.44	7.247 g
KCl	2.0 mM	74.55	0.149 g
KH2PO4	1.25 mM	136.1	0.170 g
MgSO4 or MgSO4*7H2O	2.0 mM or 2.0 mM	120.4 or 246.5	0.241 g or 0.493 g
NaHCO3	25 mM	84.1	2.103 g
CaCl2	2.0 mM	111.0	0.222 g
Glucose	11 mM	180.2	1.982 g

5.2. Electrode Protocol

5.2.1. Recording Electrode Protocol (E1)

Step 1: Pull in the Carbon Fiber

a. From a set of carbon fibers, isolate a single fiber.

b. Using a vacuum, draw in a glass capillary filament of 1.0MM x 0.58 mm (or .75 mm) x 4.0".

c. While holding down one end of the fiber, draw the fiber into the capillary so that the fiber sticks out at both ends of the capillary.

d. Verify with an incandescent light microscope at a low magnification (10X) that one fiber end sticks out of each side of the capillary tube.

Step 2: Pull Capillaries

a. Using a flaming/brown micro pipette puller (ModFel P-97 from Sutter Instrument Company) at #53 setting, place capillary in holder by bringing the capillary tube through the heating element gently in order not to break the heating filament and slowly tightening the nozzles for each holder alternating between each to evenly distribute tightened grip on the capillary.

b. Push the green pull button.

c. Once the capillary is split in half, use scissors to cut the melted capillary point at the instance where it exits the heating mechanism.

d. Remove the partly finished electrode and place in a safe holder with the electrode lying horizontally attached on a double-sided adhesive tape (vertically stored electrodes can easily break).

Step 3: Cut

a. Check electrodes under a 10x mag microscope at the point in which the fused glass begins to thicken.

b. Place electrode in a safe place.

Step 4: Glue

a. With electrodes prepared, the carbon fibers were fixated to the glass tube with an Epoxy resin/hardener. Mix the part A and part B epoxy at 20:5 ratio according to the specific gravity of your epoxy solutions making a 2-3 gram solution.

b. Mix the solution with a 1000 mL pipette slowly pulling the solution up and down about 10 times or until mixture is homogenous. Do not allow any air bubbles into the mixture as it will potentially cause voids in the adhesive bonds.

c. Hold the tip ends in the glue for two minutes and then place electrodes in a standing holder that can go in an oven at 65°C.

Step 5: Bake

a. Place electrodes in the incubator for 2 hours at 65°C (or according to recommended curing temperature for epoxy).

b. Let the electrodes cool overnight.

Step 6: Bevel

a. Place the electrode in the holder of the K.T. Brown Type micro-pipette beveller model BV-10 (Sutter Instrument Company) so that it is at a 40 degree angle and locate the electrode tip under the light microscope.

b. Turn on the rotating platform. Make sure the surface is properly lubricated by squirting nanopure water when necessary.

c. Gently lower the electrode to the rotating platform so that the tip touches the surface and let it sit for five to seven minutes adding more water when necessary.

d. Under the microscope check the tip edge of the electrode to make sure it has a slanted edge.

Step 7: Testing

a. Set up the microscope for electrophysiology using the MP-285 Micromanipulator (by Sutter Instrument Company), the platform of the Nikon Inverted Microscope Eclipse TE300, the Axopatch 200B Integrating Patch Clamp (by Axon Instruments Inc.) set at 700 mV, and an Apple MacBook Pro with AxoGraph X software.

b. Fill the electrode by about 3/4s with potassium chloride, KCl, use a microfil tip syringe and place in holder on stage of microscope. Ensure there are no air bubbles in the electrode by slowly pulling the microfil out of the electrode.

c. Fill a small dish with artificial cerebral spinal fluid (aCSF) and place under microscope.

d. Lower the electrode to the surface of the aCSF using reflection of aCSF surface and vision.

e. Place in the aCSF the ground electrode and check the electrode isn't overloaded and the RMS value doesn't exceed 3.5.

f. Turn on the Axograph to a blank page. Do not save anything.

g. Press start and wait 5 seconds to place a dopamine 100 ul drop next to the electrode into the petri dish.

Dopamine solution

Perchloric Acid 0.3ml (0.3N) mixed with 2.5 ml water. Then add dopamine powder 1mg/ml and complete mixture with nanopure water up to 10 ml.

h. Record on Axograph for 10 more seconds and then press stop.

i. Determine the quality of the electrode by the change in the baseline after the administration of dopamine. A several ms-wide peak with slow decay indicates a live electrode.

5.2.2. Stimulating (Puffing) Electrode Protocol (E2)

1. Take an empty glass capillary (the same ones used in E1 without the carbon fiber) and pull the electrodes using the same device and setting.
2. Once pulled, cut the ends just as in E1 before removing the new E2s.
3. Remove E2s and cut the ends of the electrodes so solution will only slowly spread out of the electrode when puffed. Under the microscope, the tip will look almost closed.

5.3. Laminin Protocol

Preparing Laminin from 1mg stock vial

1. Thaw the 1 mg stock vial that is shipped from the company.
2. Calculate from the concentration value that is written on the product information sheet how many µL = 20 µg.
3. Aliquot this concentration into an autoclaved tube and store in a -80˚C freezer.

Preparing Cell Culture Dishes with Laminin

1. From a prepared stock of laminin aliquots, remove one aliquot of 20 µg from the -80˚C freezer.
2. Thaw on ice before using.
3. Dilute the aliquot to 10 µg/mL in DMEM without serum.
4. Distribute 100 µL of the solution into the center of the glass bottomed cell culture dish (for 20 dishes).
5. Cover the dishes with lids and let sit for 1 - 2 hours.
6. Aspirate off the laminin solution using a vacuum-flask with a sterile pipet-tip on the end of a 5 mL pipet broken in half.
7. Let the plates dry under the sterile hood and store in 4˚C for a few weeks or plate cells on a semi-dry substrate after aspirating.

5.4. Tables of Averages

Table 5.5. Averages for Total Control Group

	Location (sec)	T1/2 (sec)	ISI (sec)	Height (pA)	Width (s)	Molecules (pA.s)
	17.48333333	0.007996667	3.516666667	211.0566667	0.016333333	3080837.077
1st	20.13	0.00269	2.97	119.69	0.0115	1796651.24
2nd	16.99	0.0017	4.79	246.33	0.016	2853827.811
3rd	15.33	0.0196	2.79	267.15	0.0215	4592032.18

Table 5.6. Averages for Total Placebo Responder Group

	Location (pA)	T1/2 (sec)	ISI (sec)	Height (pA)	Width (s)	Molecules (pA.s)
	14.405	0.001048	5.715	219.745	0.0063915	1476458.946
1st	18.16	0.00123	3.67	125.81	0.002786	1356438.24
2nd	10.65	0.000866	3.34	163.45	0.02031	1593351.07
3rd	14.75	0.00173	7.76	313.68	0.009997	1596479.651

Table 5.7. Averages for Total Non-responder Group.

	Location (pA)	T1/2 (sex)	ISI (sec)	Height (pA)	Width (s)	Molecules (pA.s)
	21.44	0.0038095	6.1915	99.98	10.1039	16586588.63
1st	29.4	0.00715	7.023	93.41	0.1178	32473724.35
2nd	13.48	0.000469	5.36	106.55	.0099	699452.91
3rd	0	0	0	0	0	0

Table 5.8. Column Statistics for Table of Averages for Total Cells

	Location (in sec.)	T1/2 (in sec.)	ISI (in sec)	Height (in pA)	Width (in sec)	# Molecules (in pA.s)
Minimum	0.0	0.0	0.0	0.0	0.0	0.0
25% Percentile	12.07	0.0006675	2.880	99.98	0.006343	1.039e+006
Median	15.33	0.0017	3.670	125.8	0.0115	1.596e+006
75% Percentile	19.15	0.00492	6.192	256.7	0.02091	3.723e+006
Maximum	29.40	0.0196	7.760	313.7	0.0930	2.124e+007
Mean	15.43	0.003937	4.189	159.6	0.02055	3.972e+006
Std. Deviation	7.845	0.006239	2.358	98.90	0.02810	6.604e+006
Std. Error of Mean	2.615	0.002080	0.7861	32.97	0.009365	2.201e+006
Lower 95% CI of mean	9.402	-0.0008583	2.376	83.54	-0.001042	-1.105e+006
Upper 95% CI of mean	21.46	0.008733	6.002	235.6	0.04215	9.048e+006
Sum	138.9	0.03544	37.70	1436	0.1850	3.575e+007

Table 5.9. Averages for Total C Group with Left Foot

Overall	location	t1/2	ISI	height	width	molecules
	16.13	0.00063167	10.293	229.36	0.002527	507875.0053
S1	21.24	0.00057	7.65	193.819	0.002445	430582.7684
S2	18.81	0.000394	11.37	320.91	0.002525	846635.1474
S3	13.45	0.000931	11.86	173.34	0.002612	246407.1

Table 5.10. Averages for Total PR Group with Left Foot

Overall	location	t1/2	ISI	height	width	molecules
	13.975	0.000695	4.184	258.92	0.00432	101325.902
S1	17.705	0.0004575	4.456	177.63	0.00174	1594021.04
S2	9.986	0.000481	4.254	150.61	0.00278	355048.4576
S3	14.235	0.001147	3.843	448.516	0.008444	1090908.209

Table 5.11. Averages for Total WPR Group with Left Foot.

Overall	location	t1/2	ISI	Height	width	molecules
	11.06	0.000281	0.864	100.73	0.002995	346487
S1	18.309	0.000331	1.945	159.8	0.0018	416542
S2	14.87	0.000512	0.647	142.38	0.00704	622920
S3	0	0	0	0	0	0

Table 5.12. Column Statistics for Tables of Averages Including only Events with a Left Foot

Location	T1/2	ISI	Height	Width	Molecules	#with Foot	Total original	
13.45	0.0003625	1.296	151.1	0.002145	300728	17.00	30.00	25% percentile
18.31	0.000512	7.580	191.2	0.002558	430583	34.50	60.00	Median
20.03	0.0007505	9.594	264.6	0.01454	968772	80.25	152.0	75% percentile
21.24	0.000931	10.87	326.5	0.02204	1.594e+006	137.0	152.0	Maximum
15.58	0.0005148	6.236	193.7	0.007002	608818	49.08	80.67	Mean
6.564	0.0002917	4.239	97.96	0.008722	488116	39.78	54.21	Std Deviations
2.188	9.722e-005	1.413	32.65	0.002907	162705	11.48	15.65	Std Error of Mean
10.53	0.0002906	2.978	118.4	0.0002974	233618	23.81	46.22	Lower 95% CI of mean
20.62	0.0007390	9.495	269.0	0.01371	984017	74.36	115.1	Upper 95% CI of mean

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