The Placebo Effect at the Single Cell Level

A thesis

submitted by

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ABSTRACT

Current drug trials and medical therapies would benefit greatly from being able to exclude individuals who have strong responses to placebos ahead of treatment, however not enough information about the mechanisms of the placebo effect is known at this time to be able to distinguish between subjects easily. One possible method could be genetic screening if a link can be established between a certain genotype and reaction towards the placebo effect. However, since the placebo effect is also a learned effect, it is imperative to identify biological markers that could distinguish strong responders from routine responders. A way to control for the influence of learned expectations by higher thought processing involved in the placebo effect is to eliminate that cognitive impact and adopt a more deterministic approach. We chose to test single cells using a modification of classical conditioning to examine whether they can respond to the placebo effect. If a difference is found on a single cell level, it will reveal more about how much of the placebo effect is learned and how much is influenced by changes at the cellular and molecular level.

Previous neuroimaging experiments have shown that placebo-related expectations induce similar responses in dopaminergic release in the midbrain comparable to dopamine response to reward processing. Two inbred strains of rats, obesity prone (OP) and obesity resistant (OR), with differing basal and elicited levels of central dopamine, were found to have significantly different sensitivity to the placebo effect. Using these rats' neuroendocrine cells, we aimed to first establish a novel method of testing whether or not a placebo effect can exist on the cellular level alone, without the moderation of higher brain functioning. We also examined whether a difference in basal dopamine

levels influences the strength of this effect on a cellular level as it did while part of the whole reward pathway. We report significant differences in dopamine release of cells induced by the placebo effect in both OP ($t = 2.632$, $p = .013$) and OR rats ($t = 4.359$, $p < .001$), with a much greater significance in the OR group. This corroborates previous studies linking higher basal dopamine levels to the strength of the placebo response, as well as implies that individual cells have the capacity to alter behavior based on expectancy.

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LIST OF ABBREVIATIONS

CNS Central Nervous System

CS Conditioned Stimulus

CR Conditioned Response

DMEM Dulbecco's Modified Eagle Medium

OP Obesity Prone

OR Obesity Resistant

PNS Peripheral Nervous System

LED Light Emitting Diode

US Unconditioned Stimulus

UR Unconditioned Response

INTRODUCTION

Background of the Placebo Effect

The placebo effect is a puzzling phenomenon in medicine. It seems to be, at least partly, a form of classical conditioning where the patient pairs a stimulus, such as a pharmaceutical drug or other therapy, to the effect of that treatment, usually with alleviation of symptoms. The placebo itself is an inert substance that has no inherent therapeutic value such as a sugar pill, sham surgery, or other inert treatments. The placebo effect is a response to this inert treatment that reproduces the patient's response to its active counterpart. By definition, the placebo effect is said to have occurred when there is a positive effect on the patient given an inert substance that is stronger than a reaction seen in a null control, such as when the patient is taking no medication.

Though considered by many to be solely an artifact in medical research, if properly applied the placebo effect can be utilized to potentiate the effects of a true therapy. Using an inactive drug as a placebo can cause the patient to experience benefits of the drug without adverse side effects if the patient is aware of the positive effects of the therapy but not the negative effects. Perhaps more surprising, patients who knowingly receive placebos can still have a better therapeutic reaction to the placebo compared to no treatment (Kaptchuk TJ. et al., 2010).

Additionally, current Food and Drug Administration trials for drugs entering the market make use of placebo groups in testing as a control to compare the overall effectiveness of the drug. While the experimental groups are typically randomly assigned into those receiving the actual medication and those receiving a placebo, the distribution

of subjects across groups does not currently abide by scientific criteria with respect to the strength of their placebo response. Because of this, it would be critical to be able to target subjects where placebo based therapy would be viable (such as by genetic marker) so test groups remain unbiased.

A patient's reaction to the placebo effect can vary from person to person (Renna et. al, 2009). The reasoning behind this is still being investigated. It is uncertain whether predetermined factors, such as gene expression, or learned behavior plays a larger role in the variability of the placebo effect.

Relationship Between Dopamine Expression and Reward Expectation

Previous studies (Geiger et. al, 2008) have linked dopamine expression to obesity prevalence. The working theory is that dopamine is a major component of the mesolimbic reward pathway, which plays a clear role in expectation of rewards and may result in hedonistic behavior regarding food such as observed in obesity. Geiger et al. recorded the quantal dopamine release between two types of rats, those that were genetically predisposed to become obese if given a high-fat chow diet (OP) and rats resistant to weight gain (OR). They found that basal dopamine levels as well as evoked dopamine levels were reduced in OP rats, largely due to deficiencies in factors regarding the neurotransmitter's synthesis and exocytosis, such as attenuated vesicular-amine molecular transporter 2 and tyrosine hydroxylase.

As a way to compare dopamine release, the Pothos laboratory (unpublished observations) used the rat phenotypes previously used by Geiger et al. and attempted to

induce a placebo response in each group via a modified form of classical conditioning meant to replicate the placebo effect in an animal model. Classical conditioning is a method of learning where an unconditioned stimulus (US), a stimulus which spontaneously gives a response (an unconditioned response, UR), is paired with a conditioned stimulus (CS), in such a way that the application of the CS directly precedes application of the US. If done correctly, the organism learns to associate the CS with the US, eventually performing the UR just by stimulation from the CS (at this point, the UR is now called a conditioned response, CR). In an experiment done in the Pothos laboratory, they utilized a US, amphetamine (which gives them a euphoric sensation, a UR, as well as increasing locomotor activity, which was used to judge the effectiveness of the treatments) in conditioning OP and OR rat strains. Rats were conditioned to associate the amphetamine injection with a Light Emitting Diode (LED), a CS, by being exposed to the LED directly before being injected with amphetamine. After a sufficient amount of pairings, the rats were injected with saline instead of amphetamine after being exposed to the LED. If the rats successfully learned to associate the presence of the LED with effects of the amphetamine, they should behave similarly to their reaction to amphetamine (with an increase in motor activity) when given saline. This was indeed observed in the OR rats, but not the OP rats.

Argument for the Existence of the Placebo Effect on a Cellular Level

A strong candidate for the potential that a single cell learning model can be possible is found in the sea slug *Aplysia*, a simple animal whose entire nervous system averages about 20,000 neurons (Redish, 2003). Using classical conditioning, researchers

have been able to train *Aplysia* to reflexively withdraw its gills and siphon (CR/UR) when the tail is touched by pairing the tactile stimulus (CS) with an electric shock (US). After conditioning *Aplysia* would perform the reflex only from the CS. The fact that this kind of behavior can be learned with simple neural circuitry strengthens the argument that this kind of associative learning can occur at the cellular level. Furthermore, researchers were able to completely extinguish the learned association by severing the abdominal ganglion which was not an important piece of neuronal circuitry for the motor aspect of the reflex, but essential for peripheral nervous system (PNS) interneurons responsible for the conditioning in the reflex (Carew, Walters, Kandel, 1987). If a small set of neurons can be responsible for changes in behavior, it is possible that the biological behavior of PNS or central nervous system (CNS) neurons plays a large role in self-regulating adaptation to learned behavior, instead of just being triggered to alter their chemistry or connectivity by higher functioning areas of the brain.

It is conventional thinking that the placebo effect is a "top-down" process, where the cerebral cortex and higher cognitive functioning is at work, by influencing patients to respond to inert substances. The placebo effect might also arise as a "bottom-up" process, where the feedback of many variables, such as changes in sensory organs and localized hormonal concentrations, is aggregated by higher cognition to create a false sensation of a therapeutic effect. These two methods of learning have been widely explored and both are thought to play a role in how quickly and broadly organisms can learn varieties of rules and expectations (Sun, Zhang, 2003). The mesolimbic reward pathway is most likely crucial to this difference. If top down, a patient who takes a placebo expecting therapeutic results would be receptive to any positive change in their health, which

induces priming and sensitization in the reward pathway. If bottom up, the reward pathway is constitutively hypersensitive due to genetic, molecular, or pharmaceutical variations, and its strong response to changes in the body (even if created by inert substances) after the placebo is administered is interpreted as therapeutic by the patient.

Building a single cell placebo model

One way to determine which type of learning governs the placebo effect is to completely isolate one pathway. If single cells, without the influence of higher thought processes, can react based on expectation, then it could indicate that the placebo response has aspects of a bottom-up model. Additionally, if the bottom-up model in a single cell is found valid, it may be possible to identify the variables involved in the localized placebo effect, whether they are genetic, molecular, or pharmaceutical. If isolated, these variables might then be used to help identify strong responders and perhaps induce the placebo effect in others.

Neurotransmitter cells are strongly modular cells that have the capacity to change their behavior based on subtle changes in their environment, such as ionic gradients and temperature. For example, neurotransmitter cells can differentiate extreme temperatures through the transient receptor potential (TRP) family cation channels, such as cold activation of ANKTM1 in nociceptive neurons (Story et al., 2003). This makes neuronal cells a strong candidate for testing whether or not single cells can learn and exhibit the placebo effect when linked to temperature change.

Chromaffin cells are dopamine releasing cells, analogous to dopamine releasing neurotransmitter cells found in the CNS, part of the neuroendocrine system and found in the adrenal medulla. Their mechanism for neurotransmitter release is roughly similar to a neuronal cell found in the central nervous system, in which dopamine is released into the surrounding extracellular space in quantal packets following external chemical or hormonal stimulation. Therefore, because chromaffin cells are easier to extract and culture, our basic tests reported in the current study used these, with intentions to eventually move to neuronal cultures if results were initially positive.

The experimental model of our method to replicate placebo effect on the cellular level uses classical conditioning as its backbone. Similarly to switching a paired US (amphetamine) with a placebo US (saline) while holding the CS (LED) constant, as previously reported by Pothos' Laboratory, we attempted to switch a paired US (cation secretagogues that locally depolarize the cell membrane and induce exocytosis) to a placebo US (artificial cerebral spinal fluid (ACSF) saline) while holding a CS (heat) constant. Our goal was to attempt to induce the placebo effect in a single cell after several pairings.

METHODS/MATERIALS

Experimental animals

Three 4-week old inbred obesity-resistant and three 4-week old obesity-prone rats (as used by Geiger, 2008) from Charles River Laboratories, Inc. (Cambridge, MA, USA) were housed in reverse light-dark cycle (lights off at 8:00 AM and on at 8:00 PM), with no dietary or behavioral restrictions. Cells were harvested from groups alternatively, starting with the obesity-resistant group. In general, a new rat was sacrificed every 7-10 days.

Cell Culturing and Preparation

Rats were injected intraperitoneally with a cocktail of ketamine and xylazene in a 1:1 ratio, adjusted based on the size of the animal, to induce unresponsiveness. The animals were then sacrificed and both adrenal glands removed and placed into ice cold Locke's buffer solution. Fat tissues were mechanically separated and the remaining capsules were washed and minced into several large pieces by scalpel. The tissue was then transferred to solution containing 6ml of Locke's buffer, 10mg of collagenase A, and 18mg of bovine albumin to be dissociated under physiological temperature (37ºC) heat and stirring for 70 minutes. Additionally, every 10 minutes, the solution was gently triturated to ensure the reagents were distributed evenly. The dissociated cells were then centrifuged for five minutes. The supernatant was removed and the cell pellet was re-suspended in physiological temperature Locke's buffer and centrifuged once more, after

which the supernatant was again removed. Then, the pellet was suspended in physiological temperature chromaffin culture medium, triturated, and distributed upon growth dishes (coated prior with a combination of 100µg of Laminin and DMEM) such that 100µl of media was distributed to each plate. These dishes were then placed in a water-jacketed incubator (37ºC, 5% CO_2 level) for 1-2 hours, after which the dishes were flooded with 2.5ml of chromaffin cell culture medium and replaced in the incubator until testing (24-96 hours after plating).

Amperometry Recording

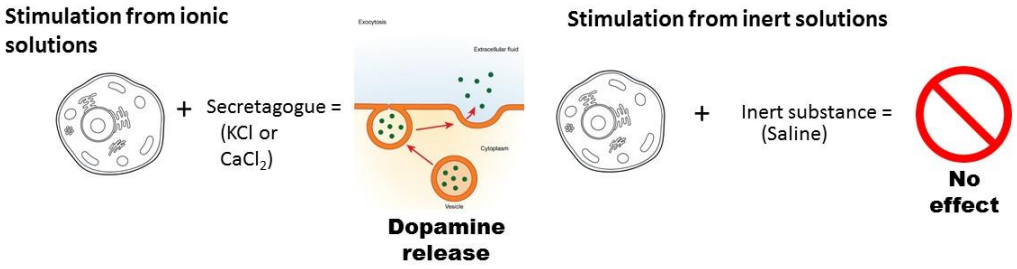


Figure 1. Chromaffin cells react to ionic solutions such as high KCl and $CaCl_2$ through inner cell depolarization and induction of exocytosis. A highly localized puff of these solutions is enough to elicit a response in the form of vesicle release, shown in on the left. In contrast, physiological saline, which is chemically analogous to extracellular fluid, applied to the cells theoretically provides relatively no reaction, as seen on the right.

Following previous successful methodology of eliciting dopaminergic signaling from chromaffin cells (Leszczyszyn, 1991), we employed carbon fiber amperometry, a cutting edge technique because of its ability to record cellular activity in real time and having high specificity for oxidizable molecules like catecholamines. 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, where internal vesicles containing dopamine fuse to the cell membrane, releasing dopamine molecules into the extracellular space in what is called a quantal or exocytic release (see Figure 1 for a graphical representation of this). These molecules were then detected by the recording amperometric electrode. Depending on the size of the quanta, highly variable amounts of dopamine could be secreted.

Recording electrodes were created by aspirating 5µm carbon fibers (Amoco) into 1mm x .75mm glass filaments and pulled using a Flaming/Brown micropipette puller (Sutter Instrument Co.). Electrodes were cut and glued, then placed in an incubator for 48 hours for glue to harden. Then, using a micropipette beveler (Sutter Instrument Co), electrode tips were beveled on one side for 5 minutes, to allow the carbon fiber to directly contact the cell and to minimize risk of rupturing the cell membrane. Before usage, finished electrodes were tested using miniscule amounts of concentrated dopamine to ensure sensitivity was moderate.

Stimulating electrodes were pulled as described, then cut under microscope enough to allow filled solutions to be pushed through. Stimulating electrodes were created in pairs, so one could be used exclusively for secretagogue and the other for saline to avoid cross-contamination. Electrode pairs were visually examined under microscope using a nonreactive dye, Fast Green (Sigma-Aldrich) to ensure the flow dynamics and amount of liquid released between the two were comparable. As electrodes were cut by hand, the amount of solution secreted per stimulation could vary slightly between different electrodes but was estimated to generally be around 20pL.

Fast Green was also included in the secretagogue and saline solutions at a concentration of 1mg/10ml during the experimentations to visually monitor flow of the secretagogue.

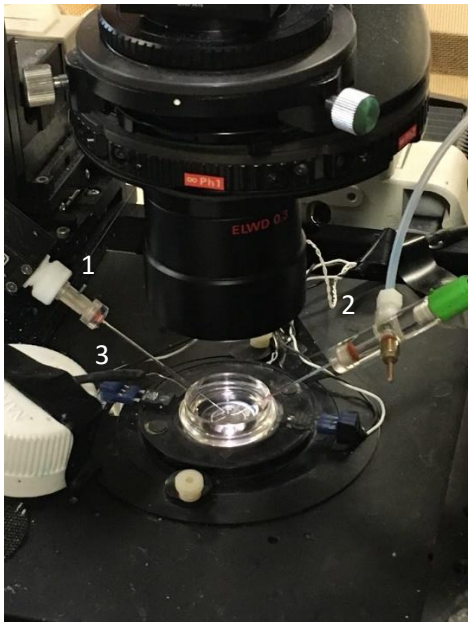


Figure 2: Amperometry setup used during experimentation. Cells were viewed under an inverted microscope. The recording electrode (left, 1) was placed on the cell to record cellular secretion of dopamine. This was achieved by the stimulating electrode (right, 2) puffing the cell with a miniscule amount of solution. A grounding wire (left, 3) was placed on the surface of the cell plate to minimize signal noise.

Plate cultures were examined under the setup shown in Figure 2, a Nikon TE300 inverted microscope. The stimulating electrode, shown in the right side of the plate, was filled with liquid and inserted into a holster connected to a Picospritzer II pressure pulser (Parker Instrumentation) which applied gaseous pressure to the uncut end of the electrode, forcing small amounts of solution through the tip. The recording electrode, shown on the left, was attached to an integrating patch clamp Axopatch 200B (Axon Instruments) which allowed for recording of oxidative currents corresponding to dopamine release. Below the recording electrode was a ground. Both electrodes were able to be precisely moved using micromanipulators (Sutter Instrument Co.) The Axopatch

was also connected to a MacBook Pro (Apple) which output the trace visually in AxoGraph X (AxoGraph), an electrophysiology recording program.

Cell plates were retrieved from the incubator within a 3-day period after plating and flooded with 1 ml of physiological saline, then transferred under the microscope. Plates were allowed to cool to ambient room temperature (21ºC) after being transferred. This temperature was not altered throughout experimentation.

Healthy chromaffin cells, which were tested preferentially, could easily be identified from other tissue by their smooth, phase-contrasted appearance. Another prerequisite for testing was that cells were also required to be attached to the plate surface, indicating that the culturing had been successful and the cell had the opportunity to survive.

Before all testing, the recording electrode was backfilled with 3M KCl and finely lowered onto the cell, enough to make contact so quantal events could be measured through vesicle release on the surface, but not enough to rupture the cell or detach it from the plate bottom. The stimulating electrode was placed 15-18μm to the right of the cell. Recording was done for at most 4-5 viable cells (that elicited at least one measurable peak from the first stimulation) in each plate.

Saline as an Inert Substance (Control Trials):

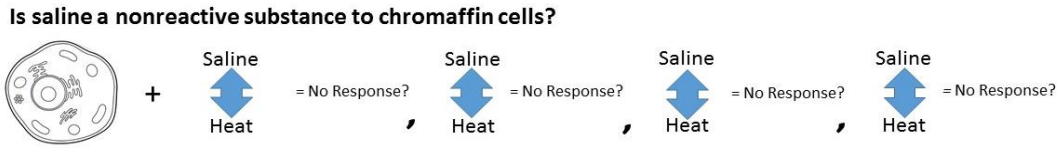


Figure 3 Experimental setup to justify the control group. Each individual cell was subjected to a "set" of four subsequent trials, where heated saline was puffed onto the cell and any exocytosis was recorded. Cells were allowed to recover for five minutes after each puff.

To test what kinds of effects physiological saline has on quantal dopamine release, each experimented cell underwent a "set" of four trials consisting of exposure to heated physiological temperature (37ºC) saline buffer four times with five-minute rest intervals between stimulations. Maintaining heated solution was accomplished by removing the stimulating electrode before every trial and backfilling it again with warmed saline. Secreted dopamine was measured by the recording electrode (See Figure 3 for an outline of this model).

A significant difference between dopamine release from secretagogues and saline unpaired in any kind of classical conditioning model would confirm that the saline has little, if any, effect and can therefore be used as an inert substance that could be reacted to in the placebo effect. If established as inert, these trials would also act as a control group to compare elicited dopamine from saline after exposing the cells to secretagogues.

Heat as a Placebo Indicator (Experimental Trials):

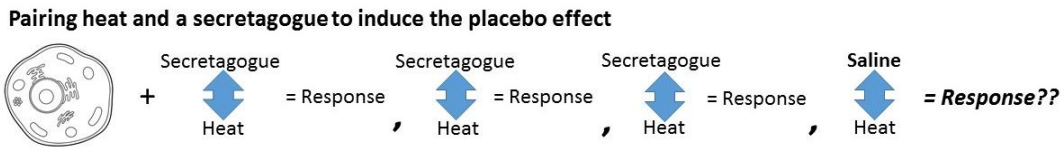


Figure 4 To induce a response to an inert substance analogous to the placebo effect, it was necessary to condition cells by associating an unconditioned stimulus (heat) to a conditioned response (dopamine release). Each individual cell was subjected to a "set" of four subsequent trials, as in the control experiment. For the first three stimulations, a heated secretagogue (KCl or $CaCl_2$) was puffed onto the cell, giving the cell five minutes of rest time between stimulations. This repetition allows for the cell to associate the CS to the UR and possibly alter its sensitivity or vesicle activity in anticipation of upcoming stimulations. Then, saline buffer, a US, was heated in a similar matter and applied to the cells once. If cells reacted the same way to saline, an inert substance, that they did to KCl/$CaCl_2$ (even if the response is diminished), the cell was considered to have successfully undergone the placebo effect.

Cells were exposed in a "set" of 4 trials which consisted of individual stimulation three times with either physiological temperature .8M KCl, 6mM $CaCl_2$, or a mixture of both. At least five minutes of rest time was given between stimulations, allowing the cell time to replenish its vesicles before the next stimulation (Ryan et. al, 1993). Then, the cells were administered an inert saline buffer for the fourth "stimulation" under the same heating guidelines, using another paired electrode to avoid cross-contamination with secretagogue (See Figure 4 for an outline of this model).

In this way, secretagogues were "paired" with heat three times to facilitate learning the association between heated solution and secretagogue. The saline administered afterwards could also be considered "paired" to heat, since the cell may have learned to associate heat with depolarization. For nomenclature purposes, the saline used in the control trials is considered "unpaired", since the cells did have any reaction to associate heat with. If cells reacted the same way to "paired" saline, an inert substance, as

to secretagogues (even if the response is diminished), the cell was considered to have successfully "learned" the placebo effect.

Data analysis:

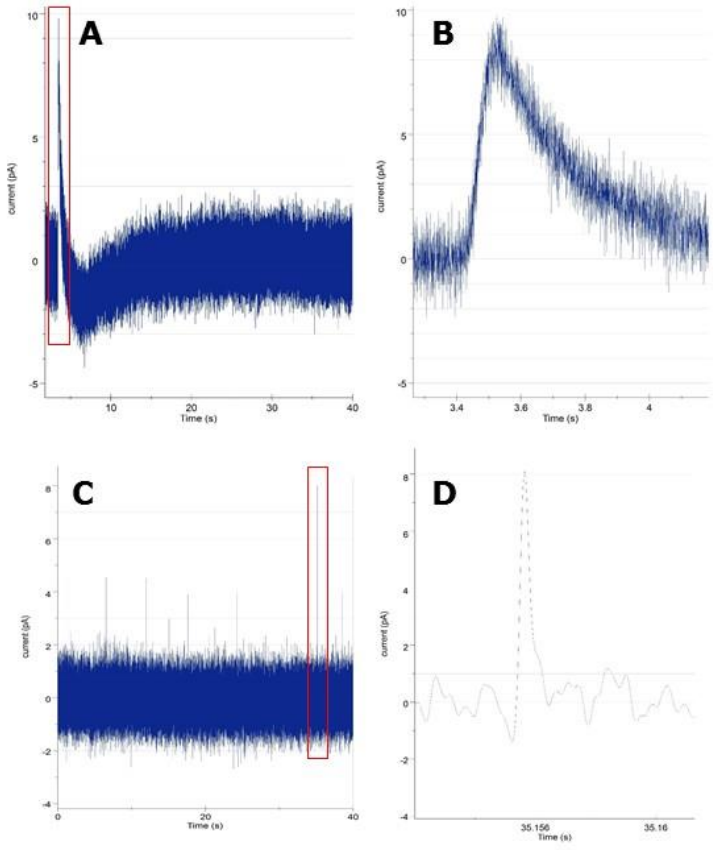


Figure 5 Samples of electrophysiology data. A: A recording from the second stimulation of a chromaffin cell from an OR rat. Of note is the single large peak that descends slowly, indicating release of several vesicles simultaneously (enlarged in B). C: A recording taken on the first stimulation of a cell from an OR rat. In contrast, this recording features several small peaks indicating release of individual quanta over the 40 seconds (enlarged in D).

Dopamine exocytosis was recorded using Axograph X. Release was recorded over forty seconds, with the secretagogue administered at the five second mark. The

sampling was digitized at 50 kHz and low-pass filtered once at 1 kHz. The threshold for a dopamine exocytosis event, where vesicles are fully released, was considered to be a peak at least three times the height of the baseline, where signaling leading directly up to the peak is normalized to zero. However, vesicle size and depolarization strength can vary, causing a release of relatively smaller amounts of catecholamine molecules. These peaks were typically much shorter in height, length, and decayed quicker. See Fig. 5 for a comparison between traces of varying size and sample of Axograph data.

Peak area (integral/AUC), amplitude (height), duration (width) and decay rate ($t^{1/2}$) were calculated. Area would indicate how much dopamine was released in a quantal packet or combination of packets. Amplitude is thought to represent the maximum flux of neurotransmitter through the respective pores, where differences in amplitude may suggest a possible difference in physical characteristics of the neurotransmitter pores in the periphery. The duration of the peak reflects the rate of degradation or recycling of neurotransmitters and vesicles. Decay rate gives an impression for the release rate of catecholamines from vesicle terminals.

Based on the area of peaks, we could also mathematically infer how many molecules of dopamine were released by each vesicle. The number of dopamine molecules recorded was determined by the equation $N = \frac{Q}{nF}$, where N is the number of molecules, Q is the integral of the peak, n is the amount of electrons transferred (two for catecholamines such as dopamine), and F is Faraday's constant (96,485 coulombs per equivalent).

Statistical analysis was run using student's t-test and 1-way unmatched ANOVA (GraphPad PRISM).

RESULTS

Statistical legend

Control Trials				
Control Trials	Trace 1 (Saline)	Trace 2 (Saline)	Trace 3 (Saline)	Trace 4 (Saline)

Experimental Trials (OP Rats)				
Exp. Trials	Trace 1 (Sec.)	Trace 2 (Sec.)	Trace 3 (Sec.)	Trace 4 (Saline)

Experimental Trials (OR Rats)				
Exp. Trials	Trace 1 (Sec.)	Trace 2 (Sec.)	Trace 3 (Sec.)	Trace 4 (Saline)

Table 1: Statistical legend indicating what groups are being compared in each section of results. The singular upper table represents data sets from the control trials, while the two boxes below is data from the experimental trials, separated into individual strains of rat when needed. Compared groups are highlighted black.

There are several permutations of testing between groups in the following section. In order to guide the reader, a simple table (shown above, Table 1) is used to highlight which control or experimental groups were compared. Any group being compared in that section is colored in black.

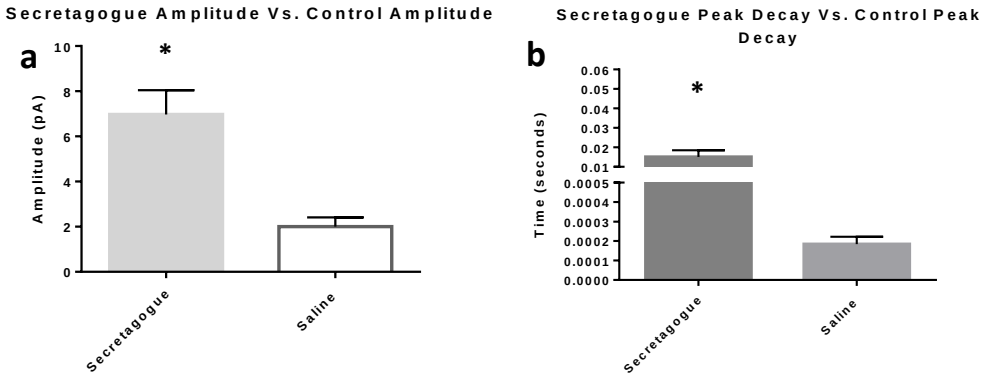
Secretagogues vs. Saline (Establishing saline as inert)

Control Trials				
Control Trials	Trace 1 (Saline)	Trace 2 (Saline)	Trace 3 (Saline)	Trace 4 (Saline)

Experimental Trials (OP Rats)				
Exp. Trials	Trace 1 (Sec.)	Trace 2 (Sec.)	Trace 3 (Sec.)	Trace 4 (Saline)

Experimental Trials (OR Rats)				
Exp. Trials	Trace 1 (Sec.)	Trace 2 (Sec.)	Trace 3 (Sec.)	Trace 4 (Saline)

Significant differences were found between control stimulations with saline and all instances of stimulation with secretagogue combined (first, second, and third traces in the experimental trial sets) in amplitude, (t = 2.14, p = .035), peak decay (t = 2.05, p = .042) peak duration (t = 2.248, p = .026), and peak area (t = 1.97, p = .05). This is graphically represented in Figure 6 as bar graphs with standard error of mean.



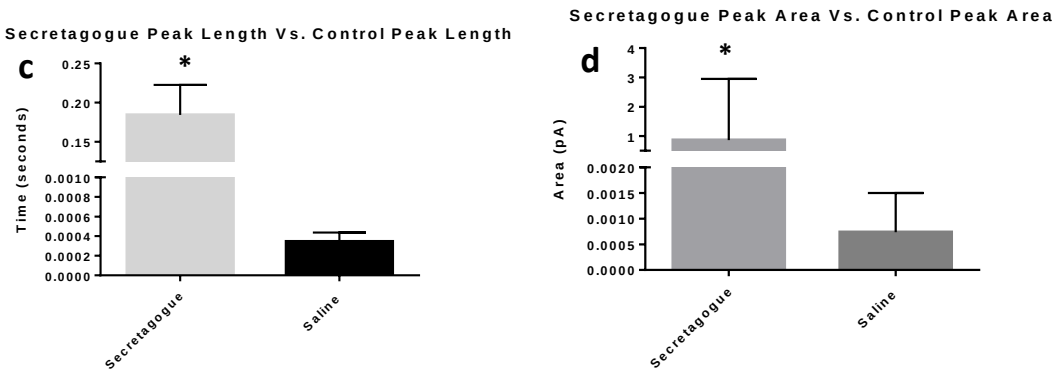


*Figure 6 Comparison between dopamine exocytosis elicited with secretagogues ($KCl/CaCl_2$) and unpaired saline as used in the control, with several parameters in peak characteristics. a: Comparison of peak amplitude (height) between secretagogue traces and control traces (t = 2.14, p = .035), showing that dopamine release by stimulation from secretagogues elicit significantly higher peaks than from 'stimulation' of saline. b: Comparison of peak decay between secretagogue traces and saline traces (t = 2.05, p = .042), showing that dopamine release by stimulation from secretagogues elicit significantly peaks that decay significantly longer than from 'stimulation' of saline. c: Comparison of peak duration (length) between secretagogue traces and control traces (t = 2.248, p = .026), showing that dopamine release by stimulation from secretagogues elicit peaks that last significantly longer than from 'stimulation' of saline. d: Comparison of peak area (AUC/Integral) between secretagogue traces and control traces (t = 1.97, p = .05), showing that dopamine release by stimulation from secretagogues elicits more dopamine overall than from 'stimulation' of saline. n = 105 for secretagogue traces, n = 23 for control traces. * indicates significant difference between both groups,* p*<.05.*

ANOVA between traces

Control Trials				
Control Trials	Trace 1 (Saline)	Trace 2 (Saline)	Trace 3 (Saline)	Trace 4 (Saline)

Experimental Trials (OP Rats)				
Exp. Trials	Trace 1 (Sec.)	Trace 2 (Sec.)	Trace 3 (Sec.)	Trace 4 (Saline)

Experimental Trials (OR Rats)				
Exp. Trials	Trace 1 (Sec.)	Trace 2 (Sec.)	Trace 3 (Sec.)	Trace 4 (Saline)

To examine if there are significant differences between traces in a same-cell "set" would give insight into the impact of multiple consecutive stimulations on exocytic behavior in a cell. For example, if traces significantly decrease after the first stimulation, it would indicate that our experimental model possibly exhausted release in cells. This comparison was done by comparing the means of the relevant parameters between experimental traces in both OP and OR rats combined. In this condition, there was no significant difference between traces in amplitude ($F = 2.32$, $p = .077$), peak duration ($F = 1.42$, $p = .24$) and total dopamine release (area) ($F = 1.55$, $p = .20$). There was a significant difference between peak decay, however ($F = 3.07$, $p = .029$). Specifically, post-hoc tukey tests reveal a significant difference between the first and fourth traces.

Further examination reveals there is a significant difference between area of traces in only OP rats ($F = 5.76$, $p = .002$). Specifically, post-hoc tukey tests reveal a significant difference when comparing the first trace to the second trace, and the first trace to the fourth trace. There were no significant differences to report within groups for OR rats alone.

OP placebo vs. control

Control Trials				
Control Trials	Trace 1 (Saline)	Trace 2 (Saline)	Trace 3 (Saline)	Trace 4 (Saline)

Experimental Trials (OP Rats)				
Exp. Trials	Trace 1 (Sec.)	Trace 2 (Sec.)	Trace 3 (Sec.)	Trace 4 (Saline)

Experimental Trials (OR Rats)				
Exp. Trials	Trace 1 (Sec.)	Trace 2 (Sec.)	Trace 3 (Sec.)	Trace 4 (Saline)

No significant difference was found between the amplitude of peaks for the OP placebo "paired" saline traces and the control (t = 1.534, p = .134), but there was a significant difference between the decay rate (t = 2.091, p = .044), the peak duration (t = 2.663, p = .012), and the area (t = 2.632, p = .013). This is graphically represented in Figure 7, and numerically represented in Table 2.

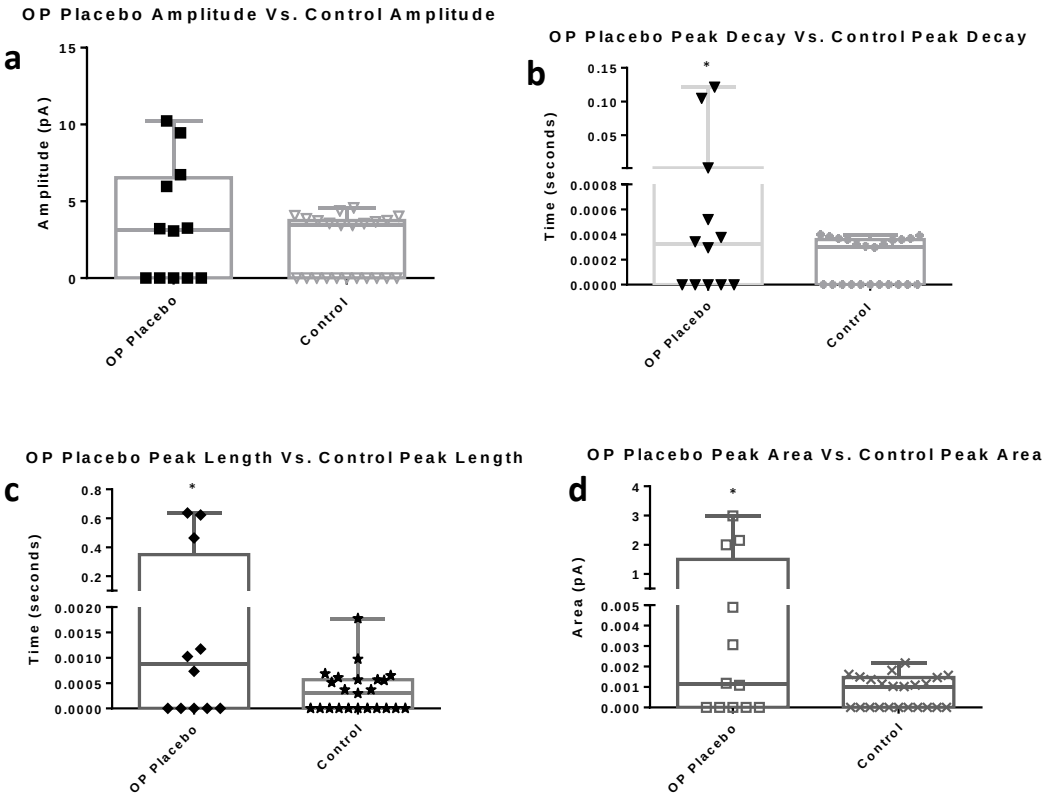


*Figure 7. Comparison between dopamine exocytosis elicited from saline after paired testing with heat and secretagogues in OP cells and unpaired saline as used in the control, with several parameters in peak characteristics. Box and whisker plots were chosen to represent this data graphically because of small sample sizes and to highlight variation from differing quantal events. a: Comparison of peak amplitude (height) between placebo traces in OP chromaffin cells and control traces (t = 1.534, ns). b: Comparison of peak decay between placebo traces in OP chromaffin cells and control traces (t = 2.091, p = .044), showing that dopamine release from saline while influenced by the placebo effect elicit peaks that decay significantly longer than from 'stimulation' with unpaired saline. c: Comparison of peak duration (length) between placebo traces in OP chromaffin cells and control traces (t = 2.663, p = .012), showing that dopamine release by stimulation from saline while influenced by the placebo effect lasts significantly longer than from 'stimulation' with unpaired saline. d: Comparison of peak area (AUC/Integral) between placebo traces in OP chromaffin cells and control traces (t = 2.632, p = .013), showing that dopamine release from saline while influenced by the placebo effect elicits more dopamine overall than from 'stimulation' with unpaired saline. n = 12 for OP Placebo traces, n = 23 for control traces. * indicates significant difference between both groups, p<.05.*

OR placebo vs. control

Control Trials				
Control Trials	Trace 1 (Saline)	Trace 2 (Saline)	Trace 3 (Saline)	Trace 4 (Saline)

Experimental Trials (OP Rats)				
Exp. Trials	Trace 1 (Sec.)	Trace 2 (Sec.)	Trace 3 (Sec.)	Trace 4 (Saline)

Experimental Trials (OR Rats)				
Exp. Trials	Trace 1 (Sec.)	Trace 2 (Sec.)	Trace 3 (Sec.)	Trace 4 (Saline)

Amplitude of peaks in the OR placebo traces were found to be significantly different from the amplitude of control peaks ($t = 2.873$, $p = .006$). Decay rate of peaks also differed significantly ($t = 3.666$ $p < .001$), as well as length ($t = 5.062$, $p < 001$) and area ($t = 4.359$, $p < .001$) (Figure 8 & Table 2)

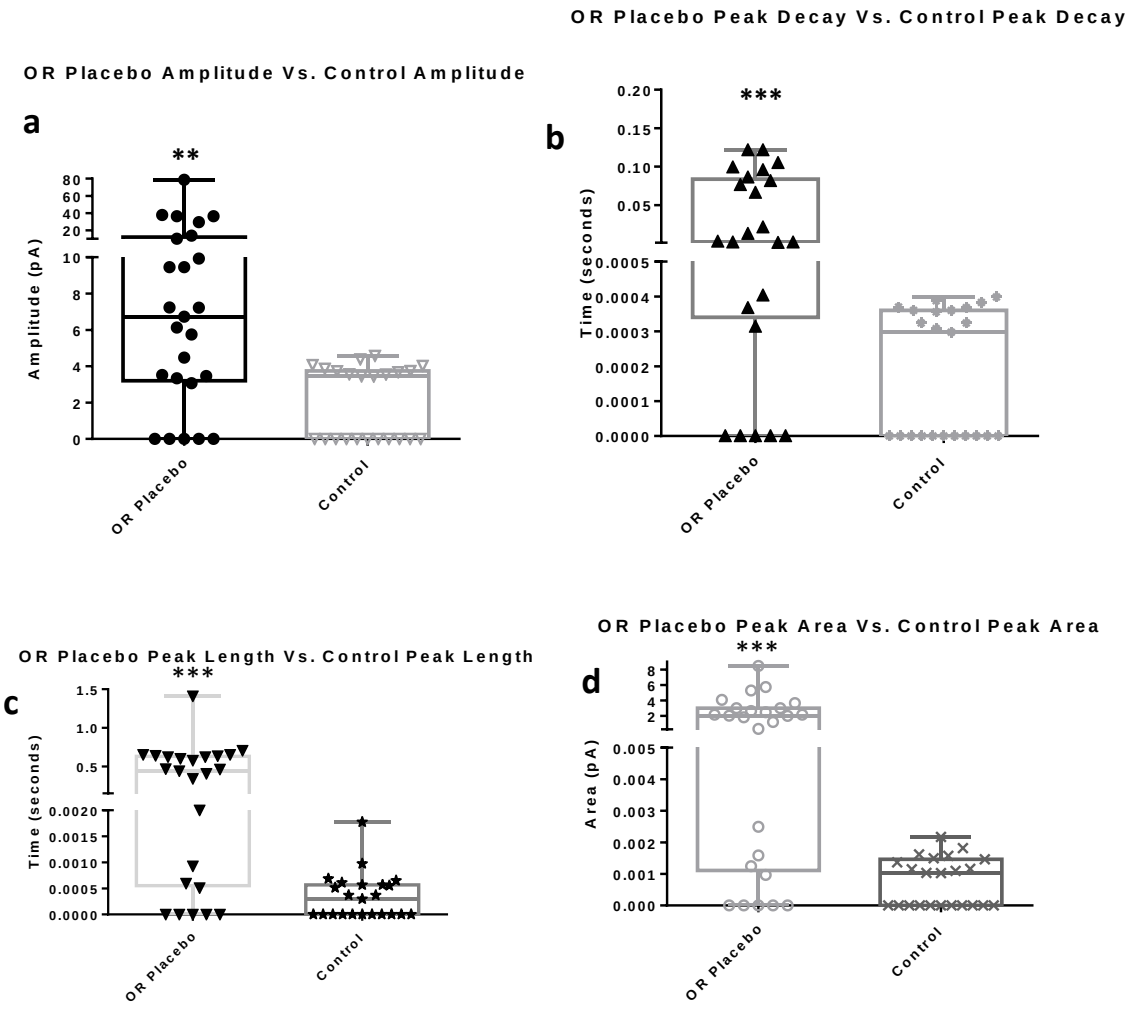


*Figure 8 Comparison between dopamine exocytosis elicited from saline after paired testing with heat and secretagogues in OR cells and unpaired saline as used in the control, with several parameters in peak characteristics. Box and whisker plots were chosen to represent this data graphically because of small sample sizes and to highlight variation from differing quantal events. a: Comparison of peak amplitude (height) between placebo traces in OR chromaffin cells and control traces (t = 2.873, p = .006), showing that dopamine release from saline while influenced by the placebo effect elicits peaks significantly higher than from 'stimulation' with unpaired saline. b: Comparison of peak decay between placebo traces in OR chromaffin cells and control traces (t = 3.666 p < .001), showing that dopamine release from saline while influenced by the placebo effect elicit peaks that decay significantly longer than from 'stimulation' with unpaired saline. c: Comparison of peak duration (length) between placebo traces in OR chromaffin cells and control traces (t = 5.062, p < 001), showing that dopamine release by stimulation from saline while influenced by the placebo effect lasts significantly longer than from 'stimulation' with unpaired saline. d: Comparison of peak area (AUC/Integral) between placebo traces in OR chromaffin cells and control traces (t = 4.359, p < .001), showing that dopamine release from saline while influenced by the placebo effect elicits more dopamine overall than from 'stimulation' with unpaired saline. n = 25 for OP Placebo traces, n = 23 for control traces. * indicates significant difference between both groups, p<.05, ** indicates p<.01, *** indicates p<.001.*

OR placebo vs. OP placebo

Control Trials				
Control Trials	Trace 1 (Saline)	Trace 2 (Saline)	Trace 3 (Saline)	Trace 4 (Saline)

Experimental Trials (OP Rats)				
Exp. Trials	Trace 1 (Sec.)	Trace 2 (Sec.)	Trace 3 (Sec.)	Trace 4 (Saline)

Experimental Trials (OR Rats)				
Exp. Trials	Trace 1 (Sec.)	Trace 2 (Sec.)	Trace 3 (Sec.)	Trace 4 (Saline)

Significant differences were found in peak area only ($t = 2.08$, $p = .045$). Peak decay ($t = 1.04$, $p = .306$) and amplitude ($t = 1.77$, $p = .085$) were insignificant, yet peak width ($t = 2.00$, $p = .053$) was only marginally insignificant. (Figure 9 & Table 2)

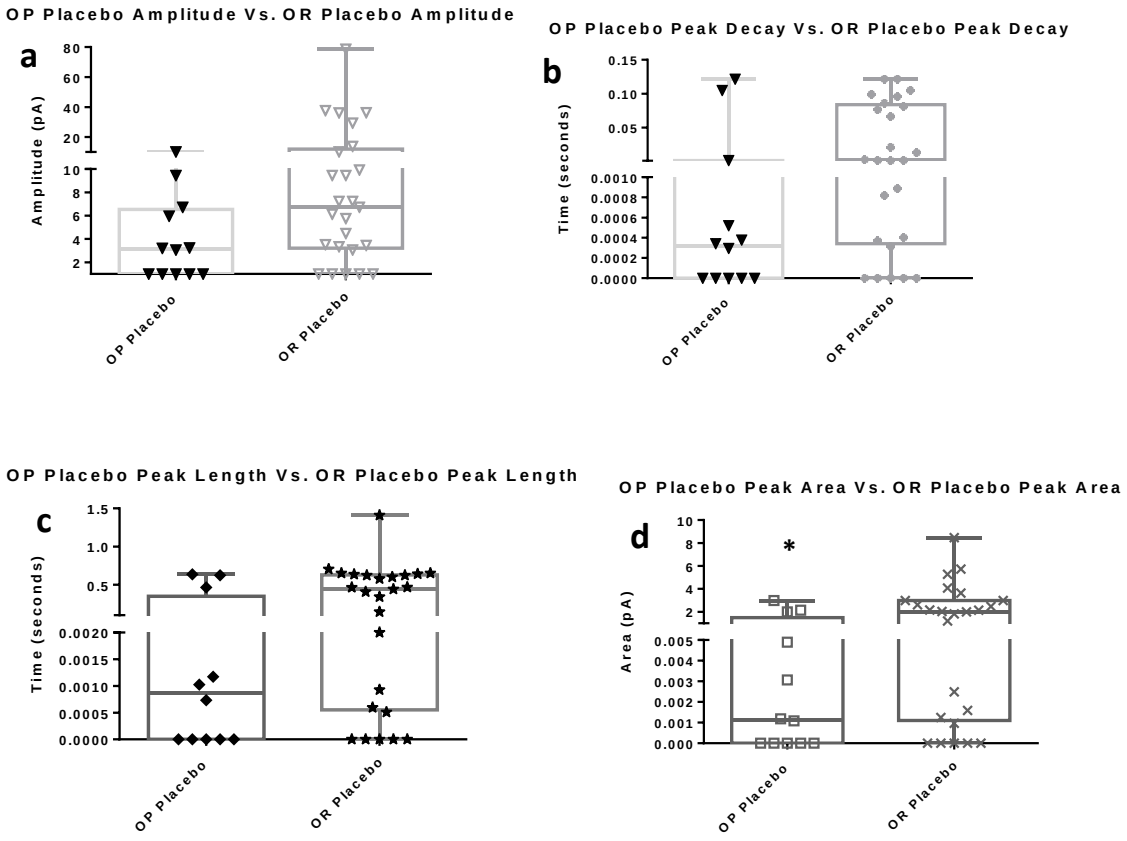


Figure 9 Comparison between dopamine exocytosis elicited from saline after paired testing with heat and secretagogues in OP cells and OR cells, with several parameters in peak characteristics. Box and whisker plots were chosen to represent this data graphically because of small sample sizes and to highlight variation from differing

*quantal events. a: Comparison of peak amplitude (height) between placebo traces in OP chromaffin cells and placebo traces in OR chromaffin cells (t = 1.77, ns). b: Comparison of peak decay between placebo traces in OP chromaffin cells and placebo traces in OR chromaffin cells (t = 1.04, ns). c: Comparison of peak duration (length) between placebo traces in OP chromaffin cells and placebo traces in OR chromaffin cells (t = 2.00, ns), showing that dopamine release by stimulation from saline while influenced by the placebo effect lasts significantly longer than from 'stimulation' with unpaired saline. d: Comparison of peak area (AUC/Integral) between placebo traces in OR chromaffin cells and control traces (t = 2.08, p = .045), showing that, compared to OP rats, dopamine release from saline while influenced by the placebo effect elicits more dopamine overall in OR rats. n = 12 for OR Placebo traces, n = 25 for OP Placebo traces. * indicates significant difference between both groups, p<.05.*

	Amplitude (pA)	Peak decay rate (s)	Peak duration (s)	Peak area (pA)
Control (Saline without pairing)	2.004 ± 0.4125, n=23	0.0001843 ± 3.792e-005, n=23	0.000345 ± 9.118e-005, n=23	0.0007380 ± 0.0001592, n=23
OP Placebo Trials	3.492 ± 1.097, n=12 (ns)	0.01917 ± 0.01275, n=12 *	0.1441 ± 0.0758, n=12 *	0.5952 ± 0.3171, n=12 * †
OR Placebo Trials	12.88 ± 3.610, n=25 **	0.03592 ± 0.009341, n=25 ***	0.3756 ± 0.07105, n=25 ***	1.997 ± 0.4389, n=25 ***

*Table 1 Matrix of statistical peak characteristics comparing saline response from placebo trials to saline response with no prior association with secretagogues (as used in the control trials), showing means, SEM, and group number, as shown graphically in Figures 7, 8, and 9. * indicates p<.05 compared to control, ** indicates p<.01, *** indicates p<.001. † indicates p<.05 when OP is compared to OR. ns = no significance in either aspect.*

It should be noted that an alternate statistical analysis would be to consider zero values as failures to release rather than zero molecules of catecholamines being released and analyze them with a non-parametric test to test how many cells from which genotype (OP vs. OR) released or not released in each phase.

DISCUSSION

Data interpretation

The definition of the placebo effect states that any response significantly higher than that seen in a negative control (no treatment) is considered a successful placebo. We showed that adrenal chromaffin cells from the OR rats were able to be influenced by the placebo effect, while OP cells were not as easily affected. This is consistent with the findings from Geiger et al., where OR rats featured comparably higher basal and placebo elicited dopamine levels in the brain compared to the OP strain, and suggests the former are more susceptible to the placebo effect. Possible mechanisms include attenuated levels of proteins involved in production, synthesis, and transport of dopamine in OP and OR rats (and cells). This would mean replenishment of dopamine vesicles after exocytosis was attenuated as well, which can inhibit or nullify "learning" in the cell. However, evidence for increased bond is stronger in vivo than in between OR cells and control cells is weakened by the fact that there is little significant difference between the experimental saline responses in OP and OR rats.

The ANOVA revealed that the first trace in the OP rats was significantly higher than most of the subsequent traces (the trace immediately after and the final, fourth trace), but this kind of difference wasn't found in OR rats. This could also be explained as a result of the reduction in time needed or reduction in capacity to replenish dopamine vesicles after exocytosis due to reduced expression of transport proteins compared to OR rats. It also indicates that a higher response to an active drug may predict a higher response to an inactive (placebo) drug even at the single cell level.

Future directions

Since we suspect the existence of genetic markers that allow for variation in strength of the placebo response in humans, it would be helpful to use single cell reverse transcriptase-polymerase chain reaction (RT-PCR) on cell groups that respond to the placebo effect and those that don't, and compare regions associated with placebo variations in humans to see if there is similar genetic variation in individual cells. [Examples: (Furmark, 2008), (Dannlowski, 2007), (Smolka, 2005) Include if time]

Different unconditioned stimuli can be used in the future. For example, dopaminergic neurons have been documented to be significantly influenced by ion-specific low frequency magnetic fields. If cells could differentiate between short magnetic stimulations, this could be used as another potential vector with which the placebo effect can be studied.

Pitfalls

The temperature of the heated secretagogues could not be measured at the point of administration due to limiting equipment. Since the liquids were transferred to the viewing plate as quickly as possible, it could be safely assumed the secretagogue were at a higher temperature than the surrounding saline media (meaning the cells should have been able to distinguish the difference in temperature). However, there is still potential for variability as the temperature of solutions exposed to the cells could be inconsistent.

Because of the "all or nothing" nature of exocytosis, there were many instances where there was no reaction from the cell, even by stimulation from a secretagogue.

Other types of experiments can usually report a failed effect as a number varying from the mean, which may negatively impact a possible effect but can be remedied easily with enough statistical power. However, our extreme method of identifying peaks as true events, where all peaks under a certain height could not be analyzed (and were counted as zero), poses questions as to whether the zero values included in our analysis should count. This can be seen in the box-whisker figures from the results section, where some points lie at exactly zero, causing a large amount of variation. In our experiment, cells that showed no response to stimulation by secretagogue in the first trace were not tested further, with the assumption the cell was nonresponsive. Another possibility not accounted for was that cells may have died in between traces due to overstimulation or mechanical pressure from a misplaced electrode. This may account for the many instances of null data. A way to remedy this would be to run one more stimulation per cell, "stimulating" with saline for the fourth trace, and then stimulating with a secretagogue once more after. If there is no response in the cell to a solution that should give a response, the cell would not have responded to a placebo anyway, so the data can be discarded. The downside of this suggestion is it would cut down on sample size, both by encouraging removal of inadequate data and by increasing the time it takes to complete a full set.

Another possible solution to this problem, to at least compare differences in the strength of the placebo effect in both rat strains, would be to either compare zero-valued events with a non-parametric test or discard null responses in a comparison between strains (to see if, *when* there is a placebo effect, one strain elicits a significantly stronger response). Only sets where there was a measurable response to placebo would be

compared between strains. However, as of now the statistical power is not high enough to allow this kind of data manipulation, but this may change with further testing.

An independent "bottom-up" model would not be able to explain how the placebo effect is not necessarily a learned phenomenon, such as when patients visit a physician only once and have relief of symptoms with no "true" intervention (though in reality they may have been conditioned by previous experiences with physicians, social influence, etc.). A possible explanation that supports how complicated studying the effect can be is that, like in other complicated behavior, both top-down and bottom-up learning may be involved. If the bottom-up process works, it likely feeds into a top-down process in a positive feedback loop. For example, a patient takes a placebo drug, which activates the reward pathway. This pleasant feeling may be mistaken by the patient as a direct effect of the drugs, who then concludes that this therapy is working. This conclusion may in turn make the reward pathway even more sensitive in the future.

Conclusion

My findings introduce an unconventional and innovative protocol to screen individual cells for susceptibility to the placebo effect. These cells can be further investigated with genetic tools to find the exact differences in gene expression that give rise to differentiated responses. This in turn can lead to further understanding of the placebo effect and its cellular and molecular "signature".

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