The Oxytocin-Induced Placebo Effect in Laboratory Animals: Implications for the Cellular and Molecular Substrates of the Placebo Response

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Abstract

Oxytocin, an endogenous neuropeptide found in mammals, is produced by the paraventricular nucleus of the hypothalamus. Its primary effects in most mammals, including rats and humans, span a wide range of physiological functions, including social behavior and bonding. Oxytocin is also involved in a host of ancillary effects including food intake/satiety, sedation, and movement. However, it is a peptide not known to directly stimulate dopamine release in the brain so it can be considered a good candidate to study the role of central dopamine in the placebo response without the apparent confounding effects of a dopamine-releasing drug. The present study considers the role of oxytocin in inducing a placebo response when the peptide is substituted by an inert substance over several days. The behavioral effects assessed include food intake and locomotor activity following either intraperitoneal oxytocin or its substitution by a saline injection. The primary hypothesis of the study was that oxytocin decreases food intake and locomotion in rats and that effect is also observed when oxytocin is substituted by saline after the animal is trained to expect a neuropeptide injection. The experiment was carried out over a 20-day trial period designed to include a baseline period at the beginning and a "placebo" period at the end. It utilized a 1 mg/kg dose of oxytocin given to male Sprague-Dawley rats. Results showed a modest decrease in both total movement and food intake after both oxytocin and placebo saline sessions and indicated that a protocol with a non-dopaminergic drug like oxytocin could potentially distinguish between strong and weak placebo responders.

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First, I'd like to express my appreciation to my fellow students who assisted me in both behavioral and electrophysiology experiments: Rulan Jiang, Aishwarya Kulkarni, and Rana Mohanna Al-Abdali. I would especially like to thank Amber Xue for her invaluable assistance and dedication in carrying out this work and data analyses. I am also grateful to Dr. Margaret Beinfeld for her assistance as my second thesis reviewer. Finally, I would like to thank my principal investigator and advisor, Dr. Emmanuel Pothos, for his continued support and encouragement in facilitating the completion of this project.

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List of Abbreviations

AVG: Average

AVP: Vasopressin

AVPR: Vasopressin Receptor

aCSF: Artificial Cerebrospinal Fluid

Exp: Experimental

FI: Food Intake

GPCR: G-protein coupled receptor

LDCV: Large Dense-Core Vesicles

NA: Nucleus Accumbens

OXT: Oxytocin

OXTR: Oxytocin Receptor

PFC: Prefrontal Cortex

PLCB: Placebo

SAL: Saline

T1: Trial 1

T2: Trial 2

TBM: Total Basic Movement

Chapter 1: Introduction

1.1 Neurobiology of the Placebo Response

Recent neuroimaging studies document the brain's response to placebo administration in subjects with Parkinson's disease, depression, pain, and other diseases (de la Fuente-Fernández 2009). These investigations validate previous observations which endorse the power of placebos to evoke objective clinical benefit and may even prolong survival. A meta-analysis of placebo-controlled trials in which mortality was a study endpoint, showed that study participants allocated to the placebo arm who adhered to the trial protocol (i.e., those who took the placebo as specified in the trial protocol) had a lower mortality rate than those who did not (Wager 2005).

From an evolutionary perspective, this observation supports the notion that placebo responses have adaptive properties that evolved from natural selection when effective treatments were lacking (de la Fuente-Fernandez, 2004). Converging evidence indicates that the expectation of clinical benefit (expectation of reward) is a major trigger for the placebo effect. Functional neuroimaging studies verify that placebo-induced expectations induce brain responses similar to those observed in reward processing (Benedetti et al., 2005). These brain responses can, by themselves, have healing effects, and can also promote "healthy" behaviors, including adherence to trial protocols. The placebo effect may also be partially mediated by conditioned behavior, whereby the pairing of a treatment with a particular stimulus leads to the same response when the stimulus is administered alone (Pecina et al., 2015). Thus, the placebo effect is deemed to

be caused by some combination of learned behaviors and expectation of reward, with the latter heavily reliant upon dopaminergic systems (Mayberg et al. 2004). Obviously, it is easier to demonstrate a placebo effect in humans than in animals since humans are informed about utilizing a particular treatment and thereby may reap the expected benefit (or side effect) of that treatment. Studying the placebo effect in rats is therefore inherently more difficult, as they cannot communicate their awareness that they are even being treated, let alone what the expected response to that treatment should be. However, certain treatments and methodologies can be leveraged. First, it is possible to shape the rat's expectations that an injection will lead to a particular response if the rat is exposed to that injection consistently for a certain number of days, incorporating the notion of Pavlovian conditioning. Second, the treatment given must cause fairly immediate benefit or harm such that the rat is able to associate the injection of the substance with its purported effects (Benedetti et al. 2005).

1.2 Previous Studies with Amphetamine

In previously performed studies in our laboratory, it was shown that a placebo effect could be established in some rats receiving an amphetamine treatment in precisely that way—they are conditioned over a number of days to an injection of amphetamine which is known to induce euphoria and to drastically increase locomotor activity. Because this is an easily measurable effect (or "reward") that is somewhat comprehended by the rat, the rat learns that an injection each day will lead to a rewarding increase in activity. After replacement of the amphetamine injection with a saline injection, it was established that about

one in every four rats would continue to respond as if it was still receiving the amphetamine, thus exhibiting a true "placebo effect." At the conclusion of these trials, the rats were sacrificed and their brains were extracted for subsequent electrophysiological experiments, which helped to generate the theory that dopaminergic systems may be involved in placebo responses. Correlations from those experiments revealed that the rats who were "strong" placebo responders also exhibited enhanced dopaminergic release and activity in the brain circuitry implicated in expectation and reward, namely the neuronal systems of the nucleus accumbens (NA), striatum, and prefrontal cortex (PFC). However, the fact that an amphetamine treatment will inherently induce significant dopamine release in the brain presents a potential confounding factor since amphetamine is known to stimulate dopamine release through its actions on vesicular monoamine transporters (VMATs) and dopamine transporter (DATs) (Seiden et al., 1993). Thus, it is not precisely known if the amplified dopaminergic response in the brains of rat placebo responders is due to a naturally enriched endogenous dopamine system, or if the difference is simply due to the drug itself. So, the ultimate question remains, since the dopamine system is probably the major player in the placebo effect, can a drug or compound that does not directly cause dopamine release also induce a placebo response in rats?

1.3 Pharmacology of Oxytocin

Oxytocin is a naturally-occurring and widespread neuropeptide, originating in the hypothalamus but which is released in areas throughout the body including the central nervous system (de Wied et al., 1993). Although its

effects have somewhat eluded scientists for many years, some of its main functions have been clarified. Some of its central and peripheral physiologic functions are related to maternal bonding, milk letdown, social interactions, grooming, feeding and satiety, and sexual behavior, among a host of other potential effects that have not yet been thoroughly explored (Gimpl et al., 2001).

Oxytocin (OXT) and its neuropeptide counterpart vasopressin (AVP) are synthesized within both magnocellular and parvocellular neurons of the paraventricular nucleus (PVN) as well as by magnocellular neurons of the supraoptic nucleus (SON) of the hypothalamus (de Wied, 1993). Whereas peripherally secreted oxytocin promotes uterine contraction during parturition and stimulates milk ejection during lactation, central release of oxytocin is implicated in both social behavior (maternal behavior, trust, emotion, social memory) and energy homeostasis (Argiolas & Gessa 1991).

In many ways neuropeptides like oxytocin act similarly to neurotransmitters, but there are some key differences in terms of their release and focal activity. Classical neurotransmitters are packaged in small synaptic vesicles that are preferentially localized at synapses (Landgraf et al., 2004). Peptides are stored in large dense-core vesicles (LDCV) which tend to be distributed in soma and dendrites as well as at nerve endings. Though both can be released by calcium-dependent exocytosis, exocytosis of synaptic vesicles requires a rise of intracellular calcium in the narrow proximity of presynaptic calcium channels, whereas peptide release is triggered by smaller but broader increases in intracellular calcium (Landgraf et al., 2004). Such changes in intracellular

calcium could be brought about by high-frequency stimulation. Thus, whereas low-frequency stimulation causes an increase in calcium at the presynaptic membrane and will trigger release of classical neurotransmitters, a more diffuse rise in intracellular calcium favors peptide release (Landgraf et al., 2004). A large number of electrophysiological studies have already been performed on acute neuromodulatory effects of peptides, including oxytocin and vasopressin. At first glance the effects seem diverse and dispersed in many regions, without a clear organizational pattern (Devost, 2008). Nevertheless, it may be possible to group some of these regions by considering them as part of neuronal circuits that underlie similar functions. Unlike conventional neurotransmission, a peptide does not simply excite or inhibit an electrically excitable cell, but is rather involved in altering the effects of other events occurring at the cell (McCarthy & Altemus 1997).

Oxytocin binds primarily to its only oxytocin receptor (OXTRs) although shows weak affinity towards vasopressin (AVP) receptors (Stoop 2012). OXTRs are centrally expressed in regions throughout the brain and spinal cord including within the hypothalamus, nucleus accumbens, amygdala and ventral tegmental area. AVP receptors (AVPRs) and OXT receptors belong to the G-protein-coupled receptor (GPCR) superfamily (Landgraf et al. 2004). Upon OXT activation, OXTRs are phosphorylated by GPCR kinase-2, bind beta-arrestin, and are endocytosed via clathrin-coated vesicles (Chatterjee et al. 2016). OTRs can reversibly switch between opposite states of affinity for agonists and antagonists depending on the presence of magnesium cations and specific interactions with

membrane cholesterol. In the rat specifically, OXTR expression can be increased by estradiol as well as by withdrawal of progesterone at constant estradiol levels (Argiolas & Gessa 1991).

As already mentioned, the functions of oxytocin are diverse and widespread. While historically considered a prosocial hormone, research shows that oxytocin promotes both positive (i.e. maternal behaviors, pair-bonding, altruism, trust) and negative social interactions (i.e. aggression, territoriality) depending on the context. Experimental uses of oxytocin indicate this neuropeptide enhances the perception of social cues, heightens cognitive processing of social information, and increases effort devoted to social engagement (Gimpl et al., 2001). In this study, we will be administering high-dose oxytocin to rats in order to primarily manipulate food intake in mildly fasting rats, as well as to note its impact on multiple facets of locomotion, including both fine and course movements (Uvnas-Moberg et al., 1994).

OXT acts as a "satiety hormone" in animals since both peripherally and centrally administered OXT reduces feeding. Feeding behavior is regulated by a variety of central and peripheral systems, and by extension, a number of orexigenic and anorexigenic entities in the hypothalamus. Within the central nervous system, neuropeptide Y, galanin, ghrelin, and melanin-concentrating hormone stimulate appetite, while corticotropin-releasing factor (CRH), bombesin, neurotensin, calcitonin, cholecystokinin and melanocortins inhibit food intake. In addition, anorexia-inducing substrates lead to pituitary OXT secretion and subsequently to reduced food intake (de Wied 1993). This suggests that both

nausea and satiety activate a common oxytocinergic pathway within the hypothalamus that controls the inhibition of digestion. PVN neurons in general, and OXT projections in particular, could act to modulate the activity of vagal efferent neurons that project to the gut and inhibit the gastric motility. The profile of the feeding inhibitory activity of oxytocin seems to indicate a true anorectic effect: the latency to the first meal is greatly increased and total time spent eating is greatly reduced, indicating increased satiety (Arletti & Bertollini 1990).

Mounting evidence suggests that oxytocin in a variety of doses can affect motor behaviors in rats, ranging from fine behaviors like grooming to total movement. Central administration of OXT in rats elicits dramatic behavioral excitation such as stress-induced escape, scratching, and pronounced grooming activity. The ability of OXT to facilitate grooming involves activation of dopamine receptor-mediated neurotransmission in the mesolimbic pathway. However, endorphins may also play a supplementary role in neuropeptide-enhanced grooming (McCarthy & Altemus, 1997). Centrally administered OXT can induce or modify several forms of behavior together with the associated motor sequences. Treatment with low OXT doses leads to a decrease in peripheral locomotor activity, whereas increasing doses of OXT induces sedative effects as indicated by a suppression of locomotor activity. A maximal effect is obtained within one hour and thereafter, the behavior gradually returned to normal within 24 hours. Because the half-life of oxytocin in the central nervous system is only about 20 minutes, this indicates that, at least peripherally, there may be a

physiologic post-treatment effect that influences motor movement (Landgraf et al., 2004).

1.4 Goals of Study

This study will first examine and confirm that oxytocin does in fact affect food intake and/or locomotor activity in rats, which will be assessed by two trials. In the first trial, all rats will receive oxytocin over a 12-day conditioning period and then receive saline for 5 days, while in the second trial, all rats will receive saline for the same 12-day period and continue receiving saline for the final five days. Assuming that a measurable difference can be established between saline-treated rats and oxytocin-treated rats in either or both of these modalities (food intake and locomotion), the next step is to determine whether any of the oxytocin-receiving rats (in the first trial) continue to respond to saline as if it were still oxytocin. In other words, the goal is to determine whether rats are able to exhibit a placebo response with oxytocin, the same way that some rats can exhibit a placebo response with amphetamine. Finally, in-vitro electrophysiology will be performed on the sacrificed rats to evaluate the differences in dopaminergic neurotransmission in key brain regions.

Chapter 2: Materials and Methods

2.1 Behavioral Experiments

The behavioral component of this experiment consisted of 2 separate 20-day trials with 2 different populations of 7 rats each. All rats were Sprague-Dawley males

and were the same age when experiment began (about 4 weeks old). The main goal was first to assess and confirm whether oxytocin has an effect on the movement and food intake of each rat. Oxytocin would be injected intraperitoneally, which is not an ideal dosing method, since only about ten percent of the oxytocin dose will cross the blood brain barrier. As such, the doses we used were very high (1 mg/kg) to ensure that a large enough portion of the dose would be able to enter the central nervous system (Morton et al. 2012).

The first three days of each trial served as a baseline where all rats in both trials received an injection of normal saline. The following 12 days is the only part of the two trials that differed. In the first trial, each rat received a weight-based injection of oxytocin (1 mg/kg) intraperitoneally (Morton et al. 2012). In the second trial, each rat received an injection of 0.3 mL normal saline. In the final 5 days, each rat in both trials received an intraperitoneal injection of normal saline. So, the second trial served as a baseline to more substantially establish the effects of oxytocin in the treated rats.

Each day of the trial, the rats were fasted from the hours of 9:30 am to 12:30 pm. At 12:30, each rat was injected with either 0.3 mL of normal saline or oxytocin (depending on the trial and day) intraperitoneally, and then immediately placed into a separate locomotor activity cage, where activity and food intake could be measured over the next 3 hours. Normal chow food pellets were weighed and then 4-5 pellets were placed into each cage. At the end of the 3-hour period, the locomotion data was collected and the food left in each locomotor cage was weighed, such that the total food intake for each rat could be

established. It should be noted that in both trials, a light was applied as a stimulus to pair with the injection, starting on the fourth day and continuing through the placebo phases to the end. The light served as a method of operant conditioning such that the rats would associate the light with an impending injection and in trial 1, the effects of oxytocin. See Figure 1.

At the conclusion of the 20-day period in each trial, each of the rats was sacrificed (1 rat per day) in order to extract the brain and perform electrophysiological studies on the pertinent brain regions. The prefrontal cortex, the nucleus accumbens, and the striatum were chosen for electrophysiology due to the high abundance of dopaminergic projections into these areas from other regions such as the ventral tegmental area and the substantia nigra.

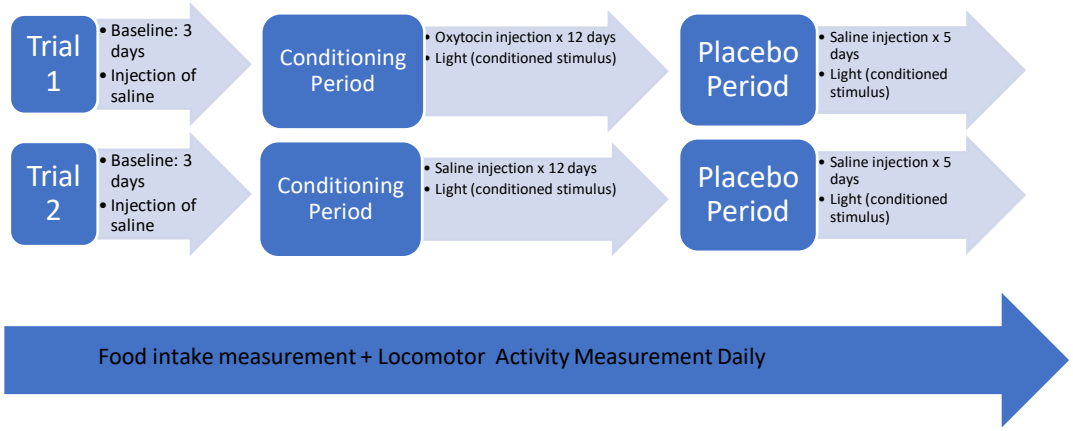

Figure 2.1 Diagram of behavioral experiment procedure. During the conditioning and placebo phases, an application of light served as the conditioned stimulus.

2.2 Brain Slice Carbon Fiber Amperometry

At the conclusion of each trial, all of the rats were sacrificed in order to extract the brain and obtain brain slices of the prefrontal cortex, the nucleus accumbens, and the striatum. These areas are the main constituents of the dopaminergic system in the brain. The rats were euthanized with an intraperitoneal injection of ketamine and xylazine, mixed in a 1:1 ratio of 40 mg each, followed by removal of the head so that the brain could be removed intact surgically. A vibratome was used to create brain slices 200-500 microns thick. The brain slices must be very thin, as this reveals neurons of interest very close to the surface. Artificial solution of cerebrospinal fluid (aCSF), which is essentially identical to endogenous CSF, was used to house the brain slices so that they could remain viable. After brain extraction, the cerebellum was removed and the brain was mounted with superglue onto the vibratome specimen disk to slice the brain and recover the desired regions of the brain (the remainder was discarded). The brain slices were placed in a chamber of oxygenated aCSF for an hour so as to give the brain tissue time to recuperate from the trauma of slicing.

The brain slices were then moved to the chamber of the electrophysiology rig, which also contained oxygenated aCSF. Using the microscope as a visual aid, it is possible to lower both the stimulating electrode and the recording electrode into the solution, such that the recording electrode's tip is placed just barely inside the neuron. Before this occurs however, the recording electrode is filled with potassium chloride (3M KCl) solution, and sodium chloride fills the recording electrode which helps to propel the signal being recorded. The recording

electrode operates at 700 millivolts when it is activated. The recording electrode transmits the neuronal signal to the amplifier, which in turn goes to a digital analogue converter and finally to the computer. The main goal was to electrically stimulate the dopaminergic terminals in the slice and acquire the response through *Axograph* software. Subsequently the curve representing the action potential can be measured for amplitude, half-life, number of molecules released, and area. Measurement of these action potentials in the noted brain regions of each rat allows total dopamine release of the neuron involved. These values would later be correlated with the behavioral data in such a way that strong placebo-responder rats and non-placebo-responder rats could be differentiated according to dopaminergic neuron activity.

2.3 Data Analysis

Data analysis comparing results from Trial 1 and Trial 2 utilized 2-sample t-tests for means (assuming unequal variance). To decrease variability, only days 4-15 (the experimental phases) from each trial were compared with regard to food intake and movement. Behavioral analysis involving only results from Trial 1 utilized paired t-tests for means (repeated measures). Ultimately, placebo response in Trial 1 was determined using an algorithm (see below) that combines all collected movement data for each rat with its corresponding electrophysiology data, namely the quantification of dopamine release in the nucleus accumbens, striatum, and prefrontal cortex. As such, each animal from the trial is assigned predicted and calculated "R" values which served as the key determinant for presence of placebo response. Food intake was considered

separately: total food intake was normalized based on individual animal weights. Placebo phase ratios and experimental phase ratios were compared to baseline ratios, and the animals with the lowest resultant values were considered stronger placebo responders. Predicted "R" values served as the expected placebo responses for each rat, and was calculated based solely on electrophysiology data from the nucleus accumbens and striatum (as seen below).

Predicted R:

$$\boldsymbol{R = (^{3\pi}/_{10})\eta e^{-\eta^2}\sin(\eta^2) + (^{5\pi}/_{20})}$$

$\eta = \frac{n_{NA}}{n_S}$, where n_{NA} is the number of dopamine molecules released in the nucleus accumbens and n_S is the number of dopamine molecules released in striatum, following an electrical stimulation.

Calculated "R" values serve as the actual placebo responses for each rat, and were calculated by integrating electrophysiology data with movement data from the behavioral component of the experiment (as seen below).

Calculated R:

$$\boldsymbol{R = A \times S}$$

$$= \frac{\sqrt{\sum_{i=1}^{36}(P'_A(t_i))^2} \times \sqrt{\sum_{i=1}^{36}(P'_P(t_i))^2}}{\sqrt{\sum_{i=1}^{36}(P'_A(t_i) - P'_P(t_i))^2}} \times \left\| \sum_{i=1}^{36} P_{P_i} \right\|$$

P: Oxytocin + light

N: Saline+ no light

PP: Saline + light

NP: Saline + No light

A(B): dPP-dP/ dN-dPP saline + no light/ Saline + light (basic movement); A(FXY): Saline + light/ Saline + no light (fine,x,y movement)(**per minute**)

P/N(B): saline +light/ Saline + no light (**every 5 minutes**)

Chapter 3: Results

3.1 Trial 1 vs. Trial 2: Food Intake and Movement

First it is necessary to verify the pharmacologic effect of oxytocin by comparing the experimental trial (trial 1) against its counterpart control trial (trial 2). Food intake and total basic movement were both significantly reduced in trial 1 rats as compared to trial 2 rats ($p<0.05$). Average basic movement of all rats during the experimental period of trial 2 (T2) was 161.719 units versus 106.008 units for all rats during the experimental (OXT) period of trial 1 (T1). Furthermore, individual rats from T2 moved significantly more than rats from T1 in all cases except for A2 (A2 from T2 moved just slightly less than A2 from T1). Food intake showed similar trends: all rats in T2 ate significantly more on average than their counterparts in T1 and ($p<.005$). Food intake averaged 3.591 grams (g) daily for T1 rats during the experimental period versus 5.687 g for T2 rats. Additionally, average overall food intake for each rat in T1 was consistently lower than food intake for each rat in T2 in all cases. Results from fine movement and total distance traveled, although not detailed here, followed similar patterns in that T2 rats generally exhibited more locomotor movement than T1 rats.

3.2 Electrophysiology Data

Interestingly, when the rats from Trial 1 and Trial 2 were compared electrophysiologically, there were some statistically significant differences between them. The only time the rats from these trials received different treatment in any way was during the experimental period where rats from T1 received oxytocin and rats from T2 received only saline. It seems that, with only one exception (amplitude within the NA), T1 rats showed reduced overall dopamine release in the brain slices studied.

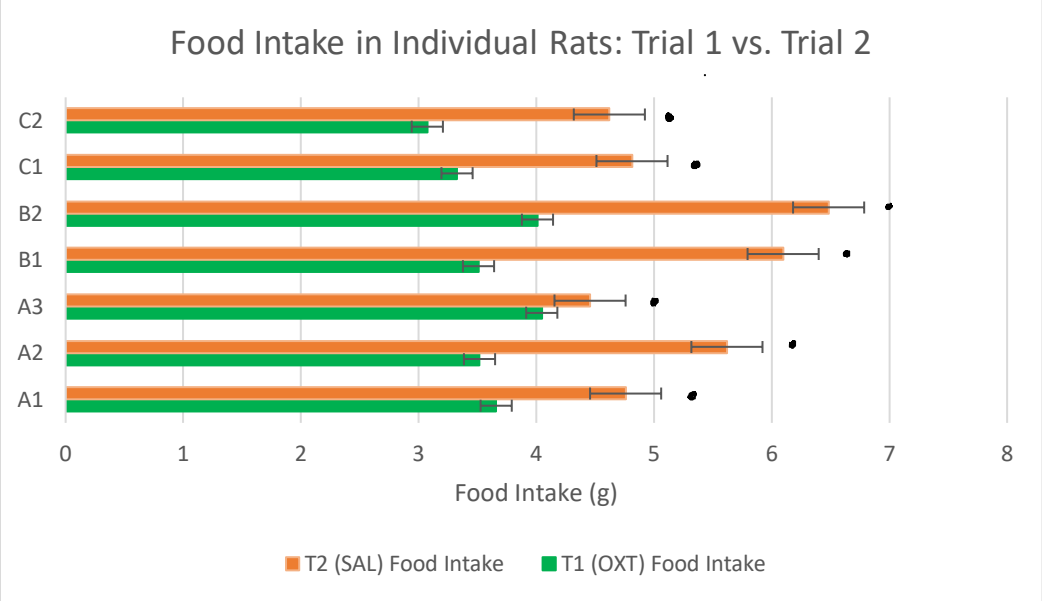

Figure 3.1 Food Intake comparison between Trial 1 (T1) and Trial 2 (T2) days 4-15. Food intake was measured in the seven rats (A1 through C2) of both trials and the totals from each trial were averaged. Food intake totals in Trial 1, where oxytocin was administered to the rats during the conditioning period, was always lower than in Trial 2, where saline was administered during the same period, though not all days individually showed statistically significant results. Dots present over bars represent statistical significance.

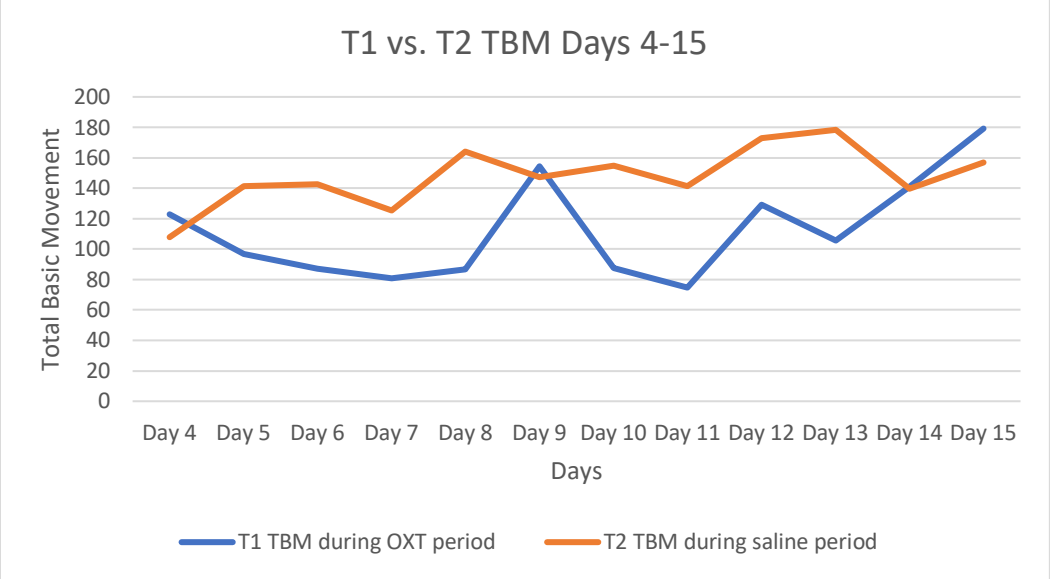

Figure 3.2 Total basic movement (TBM) over the conditioning period of Trials 1 (T1) and 2 (T2), days 4-15. Movement in all seven rats (A1 through C2) of both trials was measured over the course of three hours in locomotor cages and then these results were averaged. TBM in Trial 1, where oxytocin was administered to the rats during the conditioning period, was generally lower than in Trial 2, where saline was administered during the same period, though not all days showed statistically significant results.

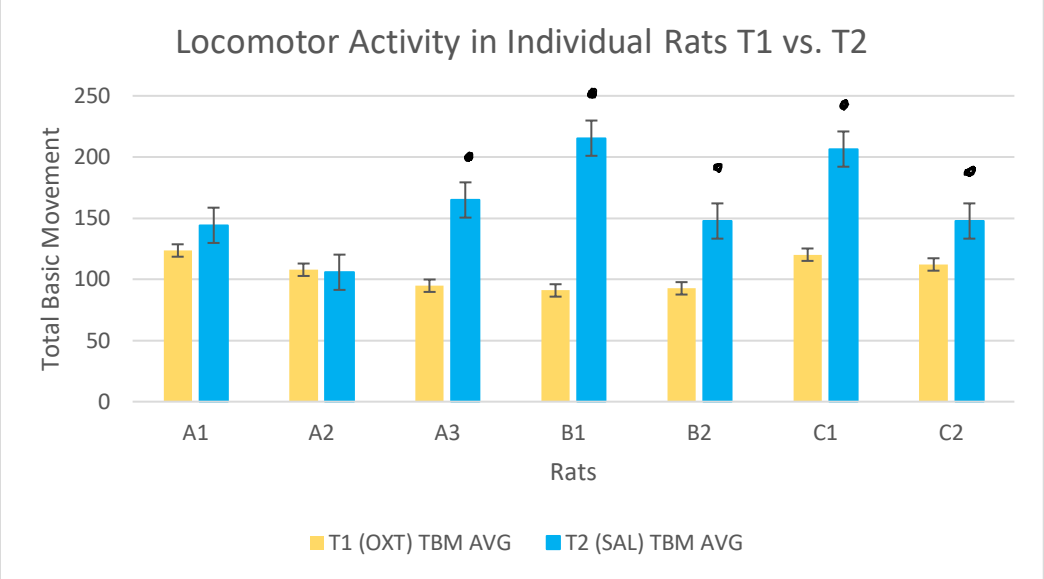

Figure 3.3 Total Basic Movement (TBM) as seen in individual rats over the course of the conditioning period, days 4-15, in Trials 1 (T1) and 2 (T2). TBM measurements were averaged for each rat from Trial 1 and then compared to its counterpart in Trial 2. TBM in the rats of Trial 1, after receiving oxytocin, tended to be lower than TBM in the rats of Trial 2 who received saline. Significant differences were seen between T1 rats and their T2 counterparts except for A1 and A2. Dots over bars represent statistical significance.

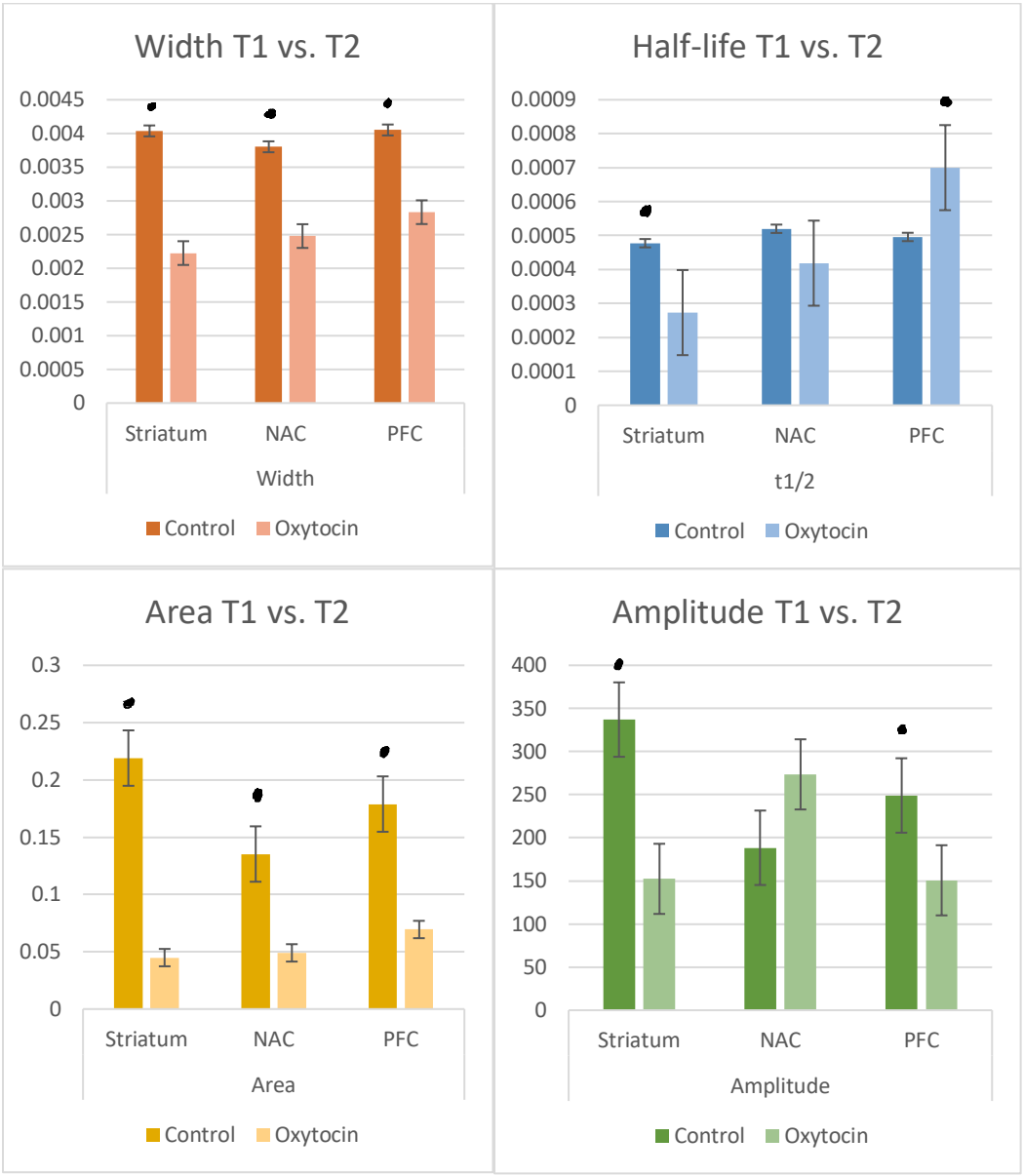

Figure 3.4 Comparison of electrophysiologic parameters between control trial and experimental (OXT) trial, T1 and T2. Data from brain slices from each area was gathered from all seven rats from each trial and averaged together. Dots over bars represent statistical significance.

3.3 Placebo Response Evaluation from Trial 1

Since the pharmacologic effect of oxytocin on food intake and movement was confirmed, Trial 1 can reliably be used for evaluation of potential placebo responses and the animals could be categorized accordingly. Considering only the raw behavioral data, there is minimal evidence that any of the rats exhibited a placebo effect at all. Food intake and movement for most of the animals during the placebo phase rebounded to baseline levels, as seen in figures. Total basic movement (TBM), while suppressed during the experimental phase, rebounded during the placebo phase to a significant degree ($p<.05$) except for A1 and B1 whose movement remained slightly lower (closer to their own movement measurements from the experimental period). Food intake for all of the animals, also quelled during the experimental phase, increased significantly during the placebo period ($p<.01$). It is important to note that, in this case, statistical significance represents lack of a placebo response due to the increases in food intake and locomotor movement after oxytocin administration was stopped. A lack of significant difference between the experimental and placebo phases could indicate a placebo response, since the measurements would likely be closer to each other. Thus, with regard to movement, there were two potential placebo responders (A1 and A2), but in terms of food intake, there were no responders based on the raw data alone. However, when the algorithm is applied (as seen in tables 1 and 2), the combination of movement with electrophysiology data reveals that two of the rats were strong placebo responders. These results are then corroborated by the food intake data, which adjusted food intake according to

animal body weights (measured weekly over the course of the trial).

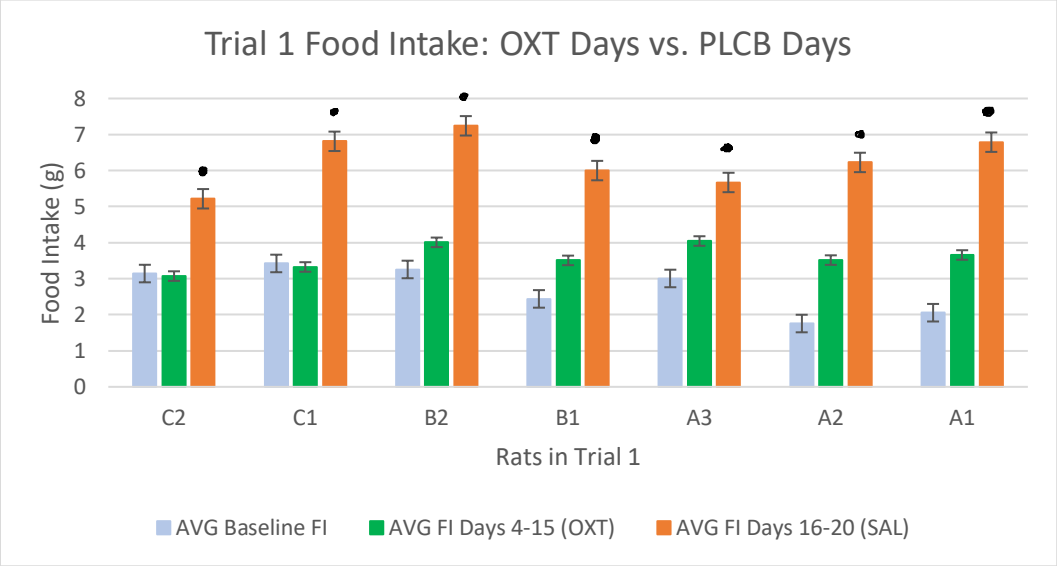

Figure 3.5 Food Intake (FI) as seen in individual rats during Trial 1. Trial is divided into three periods (baseline, OXT period, PLCB/SAL period) and food intake in each rat was measured daily during each segment. The daily FI measurements during each period were averaged together and compared here. Significance calculated based on OXT phase versus PLCB days. Dots over bars represent statistical significance of food intake during placebo (SAL) period over experimental (OXT) period.

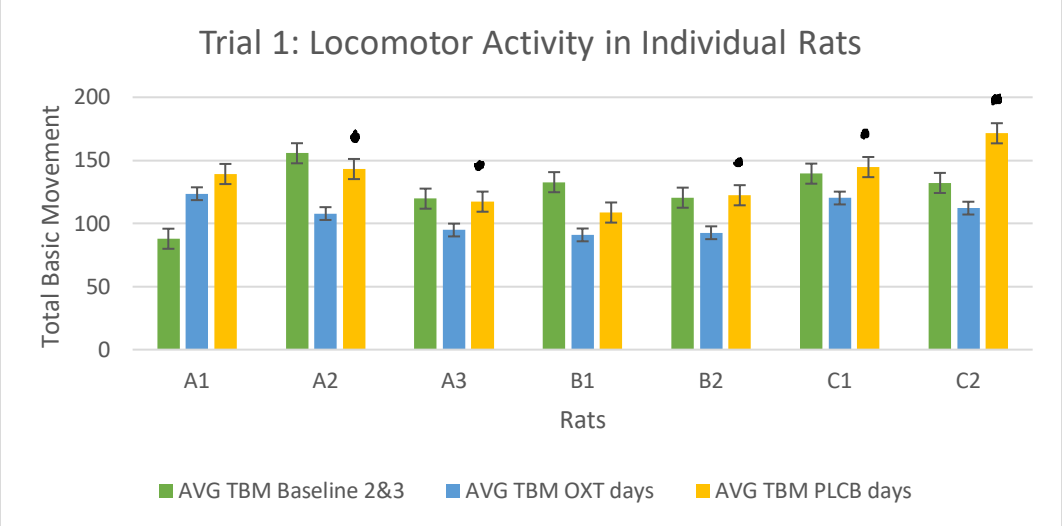

Figure 3.6 Total Basic Movement (TBM) of each rat in Trial 1, during baseline period, experimental (OXT) period, and placebo period. First baseline day was omitted from calculations for TBM due to the fact that the rats were exposed to a new environment when they were first placed in the locomotor cages and moved more than normal. Significance calculated based on OXT phase versus PLCB days. Dots over bars represent significance of OXT days versus PLCB days.

Incorporating the algorithm helps to remove extraneous variability while still factoring all experimental components into a placebo analysis, including electrophysiology and all facets of movement measured by the locomotor cages. As seen in the tables, the streamlined R-value is meant to quantitatively represent presence of a placebo response or not, where values over 1 signify a strong response and values under 1 signify a weak response. From these calculations, it is apparent that A1 and C2 may exemplify a stronger placebo response than their rat companions, and this theory is further supported with the normalized food intake data.

	Predicted R	Empirical NAC/Striatum	Calculated R	Placebo Responder?
A1	1.07715147	1	1.706534	Y
A2	0.78539816	4.660361	0.773734	N
A3	0.83387612	1.633954	0.876193	N
B1	0.76001179	2.044913	0.708748	N
B2	0.78532209	3.173015	0.833195	N
C1	0.91242656	0.576261	0.873748	N
C2	1.07804967	1.063922	1.354004	Y

Table 3.1 Table of R-values for each rat in Trial 1. R-values calculated based on the equation (see text) which factors in and combines all movement (total movement, fine movement, distance, rest time, etc.) and electrophysiology data for each animal. R-value of less than 1 indicates weak/no placebo response while R-value of more than 1 indicates strong placebo response.

	A1	A2	A3	B1	B2	C1	C2
Base FI/Wt (1)	.011313	.009258	.013508	.012074	.016098	.015323	.017262
Exp (OXT) FI/Wt (2)	.016689	.015196	.015812	.01176	.015412	.010758	.012468
PLCB FI/Wt (3)	.016637	.015062	.01258	.013712	.01695	.014809	.012591
Ratio 2/1	1.4752	1.64139	1.17049	0.97363	0.95736	0.70207	.72231
Ratio 3/1	1.47054	1.62699	0.93160	1.13566	1.05276	0.96643	0.72939
Difference (Exp – PLCB)	.0046	.0144	.2389	.162	.0954	.2644	.0071
Placebo Responder ?	Y	N	N	N	N	N	Y

Table 3.2 Table of food intake ratios for each rat in Trial 1. Rat weights were factored into food intake totals to remove weight bias/variability. Final calculated ratios from the experimental period were subtracted from the calculated ratios from the placebo period. The rats with the lowest "difference" values were considered the strongest placebo responders since their food intake changed the least between OXT and PLCB periods.

Chapter 4: Discussion

4.1 Trial Recapitulation and Conclusion

In summary, this experiment confirmed a couple of key hypotheses made, which were to assess two potential effects of oxytocin in rats, movement and food intake. Previous studies have shown in several cases that food intake is somewhat subdued when oxytocin is given to rats, while its effects on movement depend primarily on dose. Lower doses in the microgram range can increase movement

of rats but higher doses that encroach upon the milligram range usually suppress movement. The high dose of 1 mg/kg that was used in this experiment indicated that oxytocin given to rats in the first trial may curb both food intake and movement.

The first trial consisted of a 20-day period where rats received oxytocin every day for a total of 12 days and culminated with a 5-day placebo period. Several of the rats demonstrated a non-significant difference in food intake and movement between the oxytocin and placebo segments, which suggested that those rats may have been exhibiting a placebo response, albeit a very small one. It is not certain in this case whether any of the rats ever responded to placebo; the results from this experiment can suggest that two of the animals (out of seven) could have been responders, which in is range with the proportion of strong placebo responders in the human population.

4.2 Future Directions

Several components of this study are worth mentioning if these experiments are repeated in the future. The first factor for discussion would be the route of administration of oxytocin. It would behoove the experimenter to aim to give oxytocin either intracerebrally or even intranasally since the penetration of oxytocin into the brain from the blood is very low, as mentioned earlier. Intracerebral or intranasal administration would likely yield a much more substantial response, especially with regard to food intake in rats. The benefits of a larger, more obvious, response to oxytocin are two-fold. The first is that clearly it would corroborate the claim that oxytocin indeed affects food intake and

movement. The second benefit is that the enhanced responses would more clearly ascertain whether any of the rats could be placebo responders.

The second factor of the experiment that could be altered is the age of the rats. The rats in both trials were the same age, arriving when they were 4 weeks old with the experiments starting one week after their arrival. A rat reaches its full maturity and enters adulthood at about 12 weeks of age. Adolescent rats will likely eat more food with relation to their sizes due to the fact that they are still growing, and it is also possible that they may move differently than adult rats, presumably to a higher degree. This presents a confounding factor of the study, namely that the rats could vastly differ in their consumption of food and total locomotion due to their relative youth. Although oxytocin did in fact demonstrate its effects on both of these modalities, as the results showed, the rats exhibited distinctive responses that varied both individually and as a group, and even day-to-day in the same rats.

The final element of the study that could be influential to the results is using oxytocin itself as an experimental "drug." Oxytocin is inherently difficult to work with in clinical or medicinal situations due largely to its half-life in the blood of only about 5 minutes when given intravenously, possibly a bit longer if given directly into the brain. The simplest way to achieve a meaningful response was to give an extremely high dose, which allowed for enough oxytocin to enter the brain and also lengthened the total time for blood levels of oxytocin to remain

efficacious. The dosage utilized in these trials, 1 mg/kg, equated to doses close to 0.5 mg (500 micrograms) for each rat. Normal blood levels of oxytocin are in the nanogram-per-liter range, so the dose given was clearly more than high enough to establish a response, at least for about 30 minutes. However, the rats were assessed each day over a period of 3 hours after injection, so it is likely that their blood levels of oxytocin would have dissipated well before the end of the test. As stated, it may be prudent to attempt the administration oxytocin directly into the brain, since this level of oxytocin could be sustained for considerably longer. The other possibility would be to give a synthetic analogue of oxytocin, such as carbetocin. Carbetocin's half-life is on the order of at least ten times higher than that of oxytocin and mimics many of its effects (Stoop 2012).

This experiment was designed to additionally evaluate whether any of the rats would demonstrate a placebo response to oxytocin. Prior experiments which also aimed to assess placebo response utilized amphetamine as the drug and total movement in the locomotion cages as the main outcome. The results from these trials indicated that about one in four rats could demonstrate a strong placebo response, and later were able to correlate increased dopamine transmission in key brain areas of strong placebo responders. In the current experiment, it can be stated that most of the seven rats ostensibly displayed a strong "placebo response," with the exception of two animals, although the strength of the response was not assessed. The subsequent electrophysiological studies were not able to convincingly verify a correlation between dopamine neurotransmission and placebo response. There are several potential factors that obscure the

outcome of the trial's placebo component. Previous experiments, designed in a similar way to the current one, utilized amphetamine as the active drug and which produced a huge rise in the rats' movement, so the normal response of the drug was abundantly evident. Although in this trial, utilizing oxytocin as the active drug, a modest difference was noted, the effects on neither movement nor food intake were nearly as substantial as effects caused by the amphetamines. Intuitively it only makes sense that the more pronounced the original effect is, the easier it is to definitively establish the exact nature and intensity of that effect, and this improved clarity extends to placebo response as well. Simply, it was clearer to distinguish the outcome of each rat in response to amphetamine/placebo, while it was not so easy to delineate that of oxytocin/placebo. It is also important to note that the mechanism of action of amphetamine, which potently inhibits reuptake of dopamine in the brain, may have played a role in the occurrence of a placebo response.

It is hypothesized that the demonstration of placebo effect may be, in part, due to a greater presence of dopamine in the brain of said placebo responder. However, dopamine levels in the brains of both responders and non-responders would inevitably have been affected by the use of amphetamine. So it is unclear whether the electrophysiological studies done in the preceding trials measure purely intrinsic dopamine release versus dopamine release caused by the drug given: quantified dopamine levels likely reflected some combination of both. Oxytocin is not known to directly influence dopamine neurotransmission, so it

was used in this case to remove this confounding factor presented by the use of amphetamine. Here, we are reacquainted with the problem that the many actions of oxytocin are subtle and more short-lived, which made it impossible to yield a conclusive placebo response or lack thereof. If these trials were repeated, it maybe more practical to substitute oxytocin with another drug or peptide that substantially affects food intake and/or movement without directly modifying dopamine release or reuptake. A possible option that fits these criteria well would be a steroidal drug, such as prednisone or dexamethasone, which are well-known to impact food intake and are very likely to affect movement as well, without appreciably influencing dopaminergic transmission.

Overall, this study achieved meaningful results in its establishment of two ancillary effects triggered by oxytocin on both peripheral and central physiologic systems in the rat. While the primary objective of the study was well-substantiated, the secondary goal attained more tenuous results. It can be stated with confidence that intraperitoneal administration of ultra-high-dose oxytocin may decrease both total body movement and food intake in rats, as it has been proposed in numerous studies and reviews in the past. The manifestation of a placebo response is possible through the used protocol, although it would require additional results. More experiments that include some of the proposed modifications could help quantify and validate the occurrence of the placebo effect in rats, as well as identify more categorically any neurophysiologic differences in those rats that exhibit stronger placebo responses.

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