AN INVESTIGATION INTO THE EFFECT OF OXYTOCIN IN THE AMYGDALA ON GAZE FOLLOWING BEHAVIOR IN THE MALE MACAQUE

By

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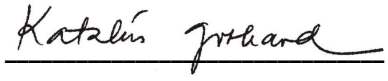

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Abstract:

Gaze-following is an essential element of social interaction, and its absence is diagnostic of autism spectrum disorder (ASD) and other neurodevelopmental disorders. Recent studies indicate that oxytocin increases attention to eyes in humans and non-human primates, and as such has possible therapeutic applications in the treatment of ASD. To test the hypothesis that oxytocin also increases gaze-following, two male rhesus macaques were given oxytocin intranasally and one was also given oxytocin injections into the nucleus basalis of Meynert (NBM) and the amygdala. The results indicate that intranasal oxytocin does increase the frequency of gaze-follows, and that this effect is likely mediated by the NBM and not the amygdala. The results also demonstrated that subjects have individual preferences (for movie monkey, gaze direction, and movement, for instance), which made them more likely to gaze-follow in response to certain movies over others. Further experiments are necessary to determine the conditions that must be met in real-life social situations that induce gaze-following in monkeys.

Introduction/significance:

Humans, and many animals, are inherently social creatures and social interaction is vital to our health and survival. In fact, deficits in normal social behavior are defining symptoms of many psychiatric and neurodevelopmental disorders, including autism spectrum disorder (ASD), borderline personality disorder, schizophrenia, social anxiety disorder, etc., which affect a large percentage of the population.

A social behavior that is one of the earliest to appear in the infancy of both humans and non-human primates is gaze-following, which is a behavior that requires an individual to fixate on the eyes of a social partner, determine the direction of his or her gaze, and make a saccade in that same direction (Emery, 2000). The planning for and execution of a gaze-following saccade requires the maturation of multiple brain systems, thus gaze-following is a benchmark of postnatal neural development and it's presence or absence is diagnostic of neurodevelopmental disorders like ASD.

Understanding the neural structures and mechanisms that underlie social interaction, beyond simply social perception, is essential to treating these disorders. While research on social perception, such as how different brain structures respond to faces and different facial expressions, is definitely fascinating and valuable, it is not as illuminating as understanding the mechanisms involved in the social interaction of one individual with another. For instance, compared to looking at the eyes, which might be governed by a perceptual mechanism (high-contrast, dynamic objects to explore), gaze-following is an elemental form of *social interaction* because the gaze behavior of an individual depends on a social partner's behavior.

Recently, researchers have been excited about one drug with the potential to treat the social deficits characteristic of ASD: oxytocin (Figure 1). In fact, studies have shown that intranasal oxytocin increases the number of saccades made toward the eyes and the total amount of time spent looking at the eyes in both humans and non-human primates (Andari et al., 2010; Guastella et al., 2008; Monte et al., 2014; Ebitz et al., 2013). Oxytocin is a neuropeptide colloquially known as "the love hormone," because it has been implicated in the formation of bonds between sexual partners in sheep, rats, rabbits, prairie voles, and humans (Kendrick et al., 1992; Hughes et al., 1987; Williams et al., 1994; Carmichael et al., 1987; Carter, 1992) and between mothers and their offspring in sheep, rats, and prairie voles (Keverne et al., 1992; Pedersen et al., 1979; Witt et al., 1990). Oxytocin is synthesized in hypothalamic nuclei (the paraventricular and supraoptic nuclei, specifically), and is released into the limbic system, including the amygdala.

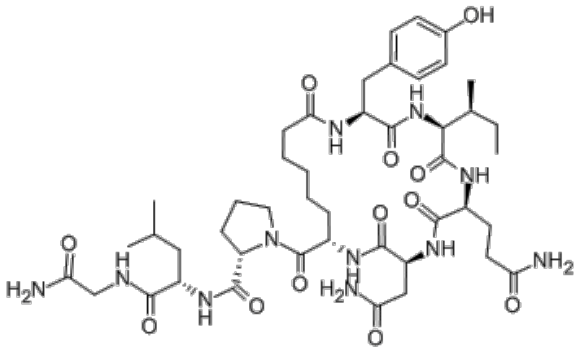

Figure 1 – Chemical structure of oxytocin.

The amygdala is a brain structure that is located bilaterally deep within the temporal lobes, medial to the hypothalamus. It is a part of the limbic system and is known to play an essential role in emotion and emotional learning (Hoffman et al., 2007). The amygdala also provides output to various other brain structures, through various pathways, and has thus been implicated in regulating attention (Anderson et al., 2003),

decision-making (Floresco et al., 2007), and modulating memory (Whalen et al., 2009; Hamann et al., 1999).

All of these functions, driven by the amygdala, are necessary for social perception, cognition (Buchanan et al., 2009), interaction, and behavior, making the amygdala an ideal target for injections of oxytocin. In rhesus macaques, oxytocin receptor mRNA is distributed in the nucleus basalis of Meynert (NBM) (Figure 2, Freeman et al., 2014), which is superior to the amygdala and sends cholinergic projections to the amygdala, including the central and basolateral nuclei. This innervation is essential for motivation, visual attention, and attentional effort (Muir et al., 1993) so the NBM is also an ideal target for oxytocin injections.

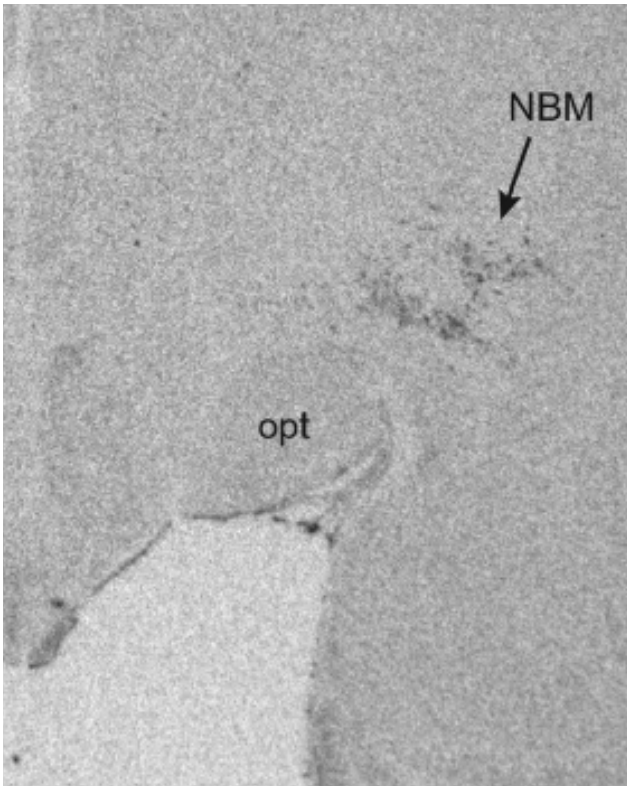

Figure 2 – Oxytocin receptor mRNA distribution (black dots) is present in the NBM and not present in the amygdala. Figure from Freeman et al., 2014.

Another method of oxytocin administration, which is more feasible for therapeutic applications, is nebulization/inhalation. Inhaled oxytocin is a nonspecific method of administration, meaning that it affects any biological structure that expresses oxytocin receptors (peripherally and centrally). Since this method of administration is how oxytocin would be, and has been (Andari et al., 2010; Auyeung et al., 2015; Anagnostou et al., 2014), delivered in patients with ASD and mice with a mutated Cntnap2 gene, which is results in ASD in humans and models the defining characteristics of ASD (Peñagarikano et al., 2015), it is important to examine the effects of inhalation on the desired behavior.

Since oxytocin has been shown to increase time spent looking at the eyes of conspecifics (Andari et al., 2010; Guastella et al., 2008; Monte et al., 2014; Ebitz et al., 2013), it might also increase the probability of detecting gaze shift in a social partner. It is also possible that oxytocin interacts with brain structures, like the amygdala, to coordinate the active engagement of the individual with others. If this were the case, oxytocin is expected to increase gaze-following independent of its effect of increasing looking at the eyes.

To test this hypothesis, the frequency of gaze-following was quantified in two adult male macaque monkeys who watched videos of other monkeys (henceforth movie monkeys) engaged in natural social behavior. To date, the majority of studies investigating the impact of oxytocin on eye-looking behavior, in both humans and non-human primates, have utilized static images of conspecific faces (Andari et al., 2010; Guastella et al., 2008; Monte et al., 2014; Ebitz et al., 2013). While these studies indicate the importance of oxytocin as a possible pharmacological treatment for social aspects/symptoms of autism or other neurodevelopmental disorders, they are limited in their scope of social behavior. The

effects of oxytocin on the social interaction aspect of social behavior can be better understood through more naturalistic, and by definition dynamic stimuli, which better represent the active nature of social interaction in real-world contexts. The use of videos of conspecifics as stimuli, instead of static images, creates more naturalistic social contexts for the evaluation of the effects of oxytocin on gaze-following behavior, while the laboratory setting controls for outside distractions and variables. Thus, eye movements and gaze direction can more accurately reveal how monkeys process and interact naturally with social stimuli.

These videos induce gaze-following in viewer monkeys and these gaze-following events do not occur randomly (Mosher et al., 2011). If a viewer monkey gaze-followed the movie monkey during a particular segment of a video, it was highly likely that he would do so during the same segment upon subsequent presentations of the same video (known as intra-viewer reliability). Moreover, if a video elicits gaze-following in one viewer, it is highly likely that it elicits gaze-following in other viewers as well (known as inter-viewer reliability). Repeated presentation of the same video, however, reduces the monkey's interest in watching the same videos (known as habituation). The phenomena of habituation, and of intra- and inter-viewer reliability of gaze-following events, provided an opportunity to determine the specific effects of oxytocin on social behavior. If oxytocin enhances interest in social stimuli in general, it is expected to counteract the habituation phenomenon, maintaining the interest of viewer monkeys in watching the same videos and increasing the likelihood of gaze-following events. Finally, it is also possible that oxytocin enhanced the active engagement of the individual with a social partner. In this case, gaze-following events are expected to increase in frequency even without enhanced eye looking.

Methods:

Subjects

Scanpaths (the sequence of eye movements and fixations) were recorded from two adult male monkeys (*Macaca mulatta*): Z and G. At the time of the study, both monkeys were between 8 and 13 years of age and weighed 10 –14 kg. Each monkey was housed alone, in a double cage, with visual access to 1-3 other monkeys in the colony. Monkey G first participated in the inhalation experiment, followed by the injection experiment approximately 9 months later. Monkey Z only participated in the inhalation experiment.

Surgery

All experimental procedures were performed in compliance with the guidelines of the National Institutes of Health for the use of primates in research and were approved by the Institutional Animal Care and Use Committee at the University of Arizona. A detailed description of the surgeries has been previously reported (Gothard et al., 2007). Briefly, to record eye movements, both monkeys were fitted with a 3-point head fixation device attached to the skull under isoflurane anesthesia.

Monkey G also received a second surgery. First, he got a pre-surgical MRI scan to determine the locations of the amygdalae in stereotaxic coordinates. Monkey G was then implanted with recording chamber above the amygdalae. A craniotomy (~8 mm in diameter) was drilled over each amygdala (Figure 5B). Between recordings the craniotomies were sealed with a silicone elastomer to maintain sterility and prevent scarring of the dura. MRI with contrast verified the orientation of the chamber relative to the amygdala.

Stimuli:

Subjects were shown blocks of 12, 10-second movies of an unfamiliar conspecific (including both males and females) ranging in age from 3–20 years. The monkeys depicted in the movies were filmed in a cage with a Plexiglas front; they were free to move around their cages and act naturally (for more details on the stimuli, see Gothard et al., 2007; Gothard et al., 2004). Each stimulus monkey was shown in three movies, displaying affiliative (lip smack), neutral, or agonistic (open-mouth threat) expressions (Figure 4). Each 10-second movie also contains segments where the movie monkey looks away and directly at the camera, creating periods of gaze that the viewer monkey perceives as averted or direct.

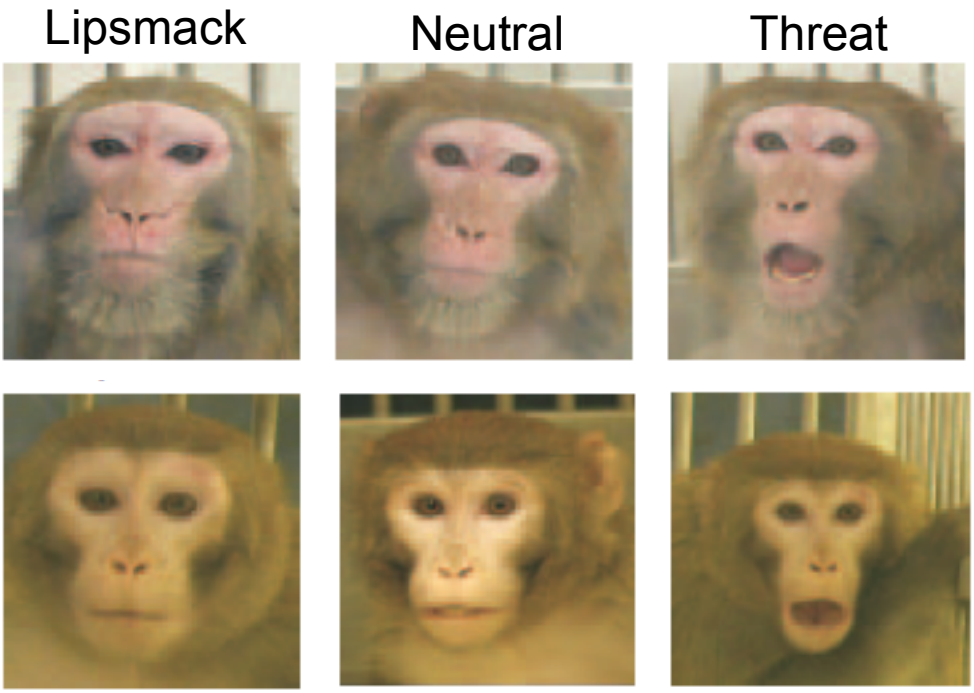

Figure 4 – Examples of lipsmack, neutral, and threat expressions exhibited by movie monkeys in the behavioral task stimuli.

Pharmacological Preparation

For nebulization, 1 mg of oxytocin powder was dissolved in 1 mL of saline in a cuvette. 85.5 µL of this solution (equivalent to 50 IU) was pipetted (using a micropipette)

into a clean Eppendorf tube and an additional 914.5 µL of saline was added. Thus, the final solution was a 1 mL solution with 50 IU of oxytocin. This procedure was repeated as necessary to make additional amounts of the solution for each experiment and each tube was kept in a freezer until needed. Eppendorf tubes containing 1 mL of saline were also kept in the freezer for the experiment. Each monkey received oxytocin via inhalation a total of 5 times and saline via inhalation a total of 5 times.

For each microinjection, 200 ng of oxytocin powder was dissolved in 2 µL of artificial cerebrospinal fluid (aCSF). Monkey G received 2 microinjections of this oxytocin solution into different regions of the NBM bilaterally and 2 microinjections into the amygdala (including the central and basolateral nuclei) bilaterally (for a total of 8 microinjections of oxytocin). Monkey G also received 3 microinjections of 2 µL of aCSF into the NBM bilaterally and amygdala bilaterally (for a total of 6 microinjections of aCSF).

Behavioral Task:

During the experiment subjects were seated in a custom-built primate chair and put in head fixation with their eyes exactly 57 cm from an LCD monitor (which spanned 37 x 28 degrees of visual angle (dva) and had a refresh rate of 60 Hz). A webcam (Logitech Quickcam, 30 fps, 8 megapixel resolution) was situated in front of the viewer monkey to capture his facial expressions (Figure 3).

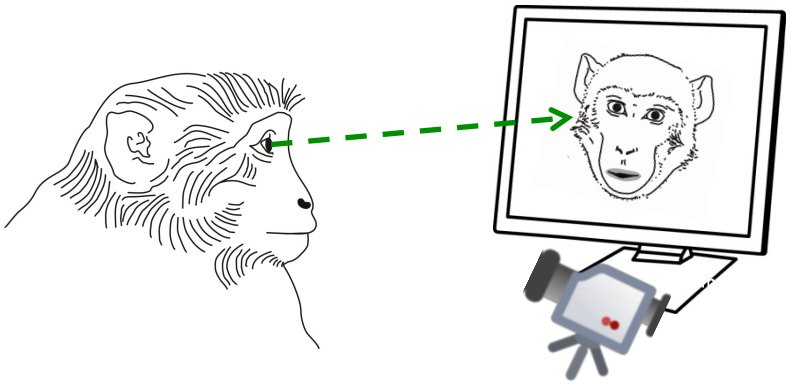

Figure 3 – Experimental setup for the behavior task

Prior to the experiment, monkeys were trained on a 9-point calibration with a precision of ±1 dva. The subject then viewed the same block of movies (in random sequence) between 3-8 times. There was a 9 second interval between movies (the screen was blank during this time). NBS Presentation software (Albany, CA) was used for cue and movie presentation. During the task, monkeys were free to look anywhere on or off the monitor. Scanpaths were recorded using an infrared camera with a sampling rate of 240 Hz (ISCAN Inc., Woburn, MA) and collected as an analog signal through a CED Power 1401 data acquisition system and Spike 2 software (Cambridge Electronic Devices, U.K.). Monkeys received reward, in the form of juice, between each block of movies.

Monkeys in the inhalation experiment then received either oxytocin or saline via nebulization while monkeys in the injection experiment received either oxytocin or aCSF via injection (see inhalation and injection procedures). Both subjects and researchers were blind to the drug being administered during every experiment. The monkey was then shown another 4 – 10 blocks of the movies and eye movements were once again recorded using the infrared camera.

Inhalation procedure

The experimenter pipetted the contents of an Eppendorf tube containing either 1 mL oxytocin solution or 1 mL saline (the experimenter was blind to the contents of the Eppendorf tube) into a pediatric nebulizer (Angel Medical Supply, Houston, TX). The nebulization mask was then held up to the monkey's face, covering his nose and mouth (Figure 5A), for 5 minutes as the solution was nebulized. An additional 1 mL of saline solution was then added to the nebulizer and allowed to nebulize for an additional 5

minutes. There was a 10-minute waiting period between the end of the nebulization and re-starting the experiment, during which time the monkey was re-calibrated.

Injection procedure

Prior to the beginning of the experiment, an MRI (Figure 5B) was obtained to determine the locations (specically the depths) of the targetted brain structures. For the injections, the experimenter inserted cannulae down to the appropriate depths and manually injected (by gently tapping the plunger) 2 µL of either oxytocin solution or aCSF bilaterally (Figure 5B). The injection was done slowly over a period of ~10 minutes. There was also a 10-minute waiting period between the end of the injection and re-starting the experiment, during which time the monkey was re-calibrated.

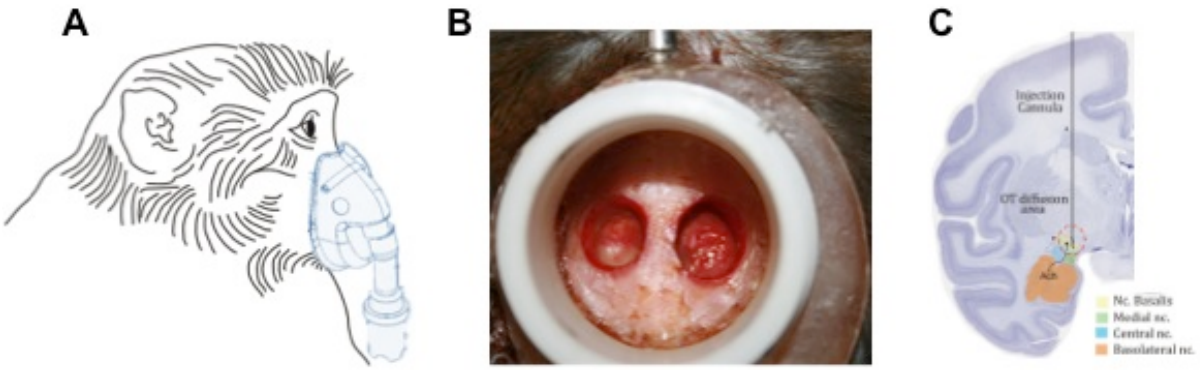

Figure 5 – (A) Schematic of inhalation using pediatric nebulizer. (B) Picture of the bilateral craniotomy that monkey G received. (C) Schematic of injection overlaid on an MRI. The red dotted circle reflects the area of diffusion of oxytocin.

Data Analysis

Scanpaths from each experiment were time locked with Presentation so they could be overlaid on top of the movies using a Matlab script. These movies were then manually scored for gaze-follows and time spent looking at the eye region of movie monkeys using Avidemux. The scorer was blind with respect to experiment day, condition, and trial. To qualify as a gaze-following saccade, the eye movements of the view monkey needed to meet

the following criteria: 1) the viewer must first fixate on the eyes of the movie monkey, and 2) the viewer must make a saccade in the direction of the movie monkey's line of sight (indicated by the movie monkey's eyes, and not the face or other facial feature) within ±30 degrees. Saccades that terminated on something in the movie (either the cage or a body part of the movie monkey) were not included as gaze-follows.

Statistics

Unless otherwise stated in the text, the following guidelines were used in the statistical analysis of data: standard analysis of variance (ANOVA) tests were used for group comparisons of more than two categories (including condition, viewer monkey, movie monkey expression, etc.). Wilcoxon rank-sum tests were used to compare data of two test conditions (e.g., oxytocin vs. control, pre-inhalation vs. post-inhalation, pre-injection vs. post-injection, etc.). In all statistical tests, significance levels were set to $\alpha = 0.05$.

Results:

Inhalation Experiments:

The findings of this study agree with those of a previous study (Mosher et al., 2011), which indicate that gaze-follows do not occur randomly. While gaze-following events occurred in approximately only 7% of movies, it was highly probable that if a viewer monkey made a gaze-following saccade during a particular segment of a movie, he would make another gaze-following saccade in the same segment of subsequent presentations of the same movie. These repeated gaze-follows occurred in both conditions, both before and after inhalation.

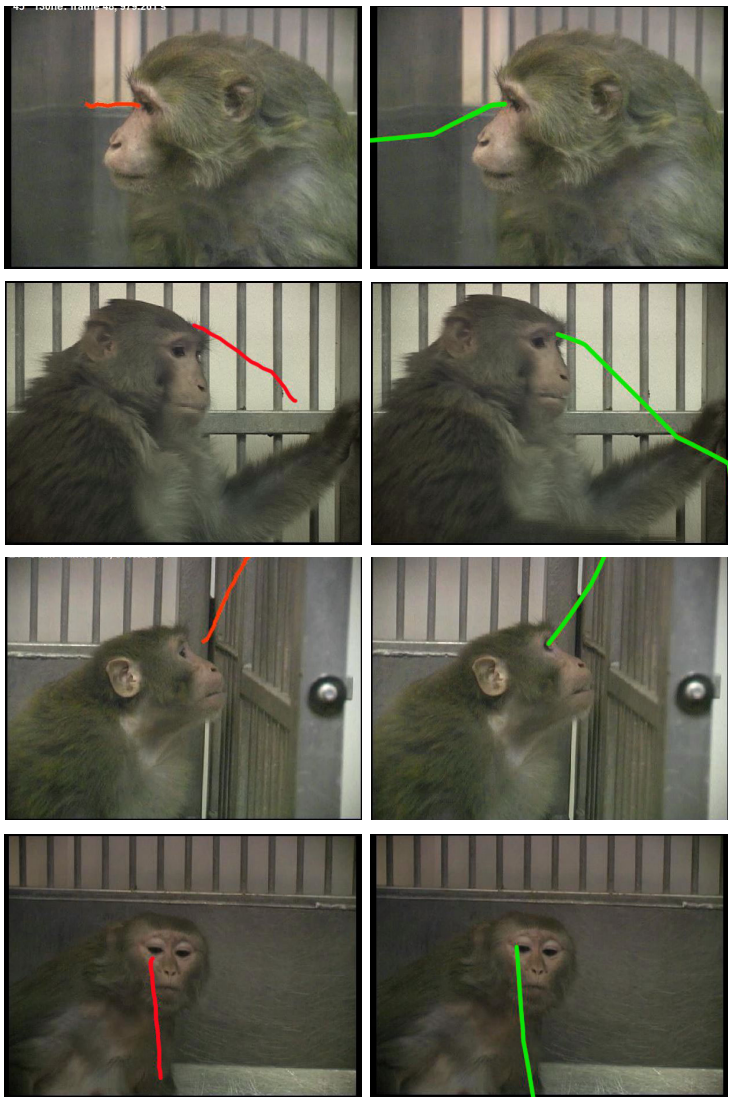

Figure 6 - Examples of frames during which both subjects made a gaze-following saccade (colored lines). Red line = monkey Z, green line = monkey G.

Additionally, if monkey Z made a gaze-following saccade during a particular segment of a movie, it was highly likely that monkey G would make a gaze-following saccade in the same segment when presented with the same movie (Figure 6).

To determine the effect of oxytocin on gaze-following behavior when administered by nebulization, scanpaths of each subject's eye movements as they viewed movies of conspecifics were scored for gaze-follows. The frequency of gaze-follows was averaged across blocks for each condition both before and after inhalation (Figure 7). The data from the two monkeys was also averaged together. On average, subjects made relatively few gaze-follows per block (an average of 1.00 ± 0.37). However, subjects did increase their frequency of gaze-following saccades after inhaling oxytocin, an effect that is not seen with saline inhalation and which is statistically significant ($p = 0.0023$) relative to the decrease in the frequency of gaze-follows after saline inhalation. A two tailed t-test between the oxytocin and saline conditions (more specifically, between the change in the frequency of gaze-follows for each condition) also demonstrated that the results were statistically significant ($p = 0.0013$).

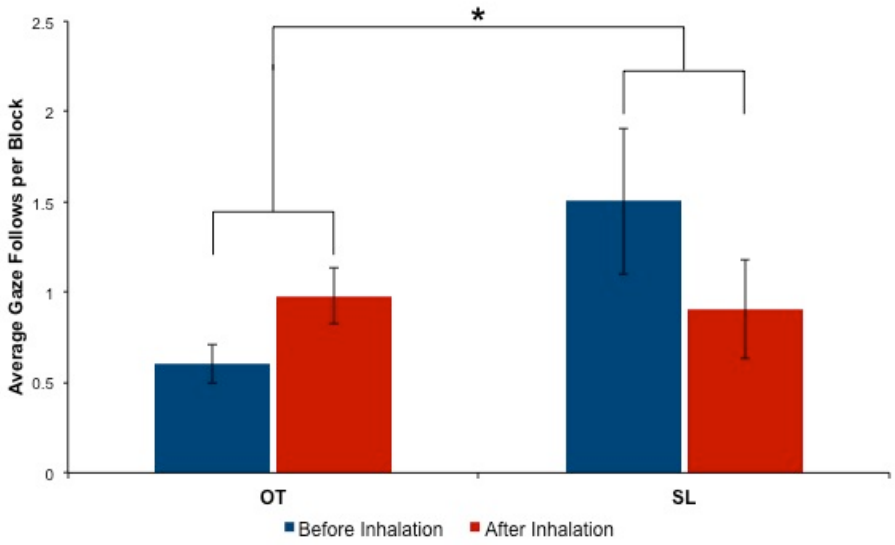

Figure 7 – Oxytocin increases the average frequency of gaze-follows relative to the decrease shown with saline due to habituation (*p = 0.0023).

Gaze-follow frequency was also plotted by block in order to visualize the trend of gaze-follows before and after the inhalations (Figure 8). Previous gaze-following data from monkeys Q, T, and V (Mosher et al. 2011) was included in this plot as another control to show the trend of gaze-follows over subsequent blocks when no inhalation is given. The trend line for this data (from monkeys T, Q, and V) slopes downward (slope = -0.365), an effect of the viewers losing interest in the movies after repeated presentations (also known as habituation). Similarly, the slopes of the trend lines representing pre- and post-saline inhalation are also negative (-0.514 and -0.638, respectively). However, in the oxytocin condition, only the trend line representing pre-oxytocin inhalation similarly slopes downward (-1.070). The frequency of gaze-follows increases after oxytocin inhalation with a slope of +0.595. This indicates that oxytocin not only counteracts habituation, but also increases the subject's engagement with the stimuli and consequently the frequency of gaze-follows.

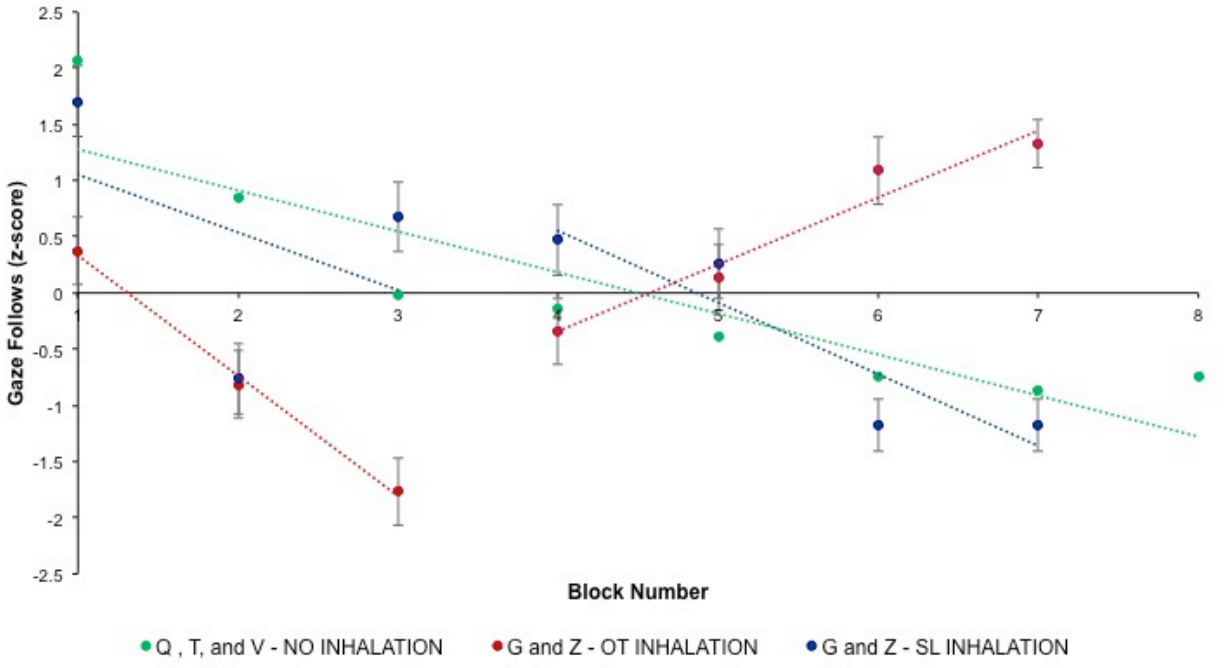

Figure 8 – Oxytocin overcomes the effect of habituation on the frequency of gaze-follows (red line), which is demonstrated by two controls: saline inhalation (blue line) and no inhalation (green line).

The data was also analyzed to determine the effect of the movie monkey's facial expression on gaze-follows (Figure 9). Each stimulus set contained three movies of four monkeys in which the movie monkey displayed a different expression (lip smacking (LS)/affiliative, neutral (NE), and open-mouth threat (TH)/agonistic). The changes in gaze-following frequency after inhalation for each condition with respect to each expression were significantly different for all expressions ($p = 0.0471$ for LS; $p = 0.0324$ for NE; $p = 0.0360$ for TH). There was also a significant increase in the frequency of gaze-follows after oxytocin inhalation with respect to threatening movies ($p = 0.0343$), even without considering the expected habituation (as seen in the saline condition). Subjects did appear to prefer neutral monkeys irrespective of condition (and also without regard to before or after inhalation).

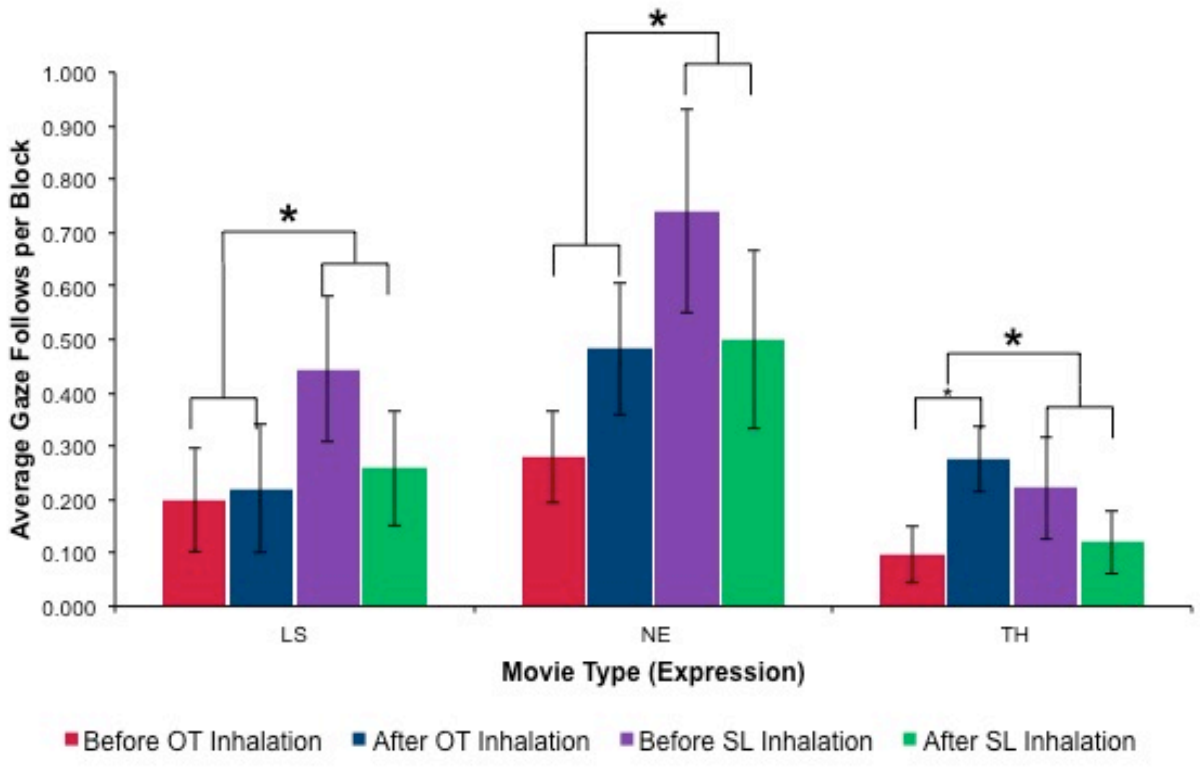

Figure 9 – Oxytocin increases the frequency of gaze-follows regardless of movie monkey's expression. This increase is significant only when compared to the decrease in frequency shown in the saline condition (due to habituation), with the exception that oxytocin itself significantly increases frequency of gaze-follows in movies featuring open-mouth threats.

Movie segments that induced a gaze-follow from the viewer monkeys were analyzed with regard to the direction of the movie monkey's gaze, which was indicated by the angle of the movie monkey's eyes and sometimes face (Figure 10). The results indicated that movie monkeys averting their gaze to 315° induced gaze-follows most frequently.

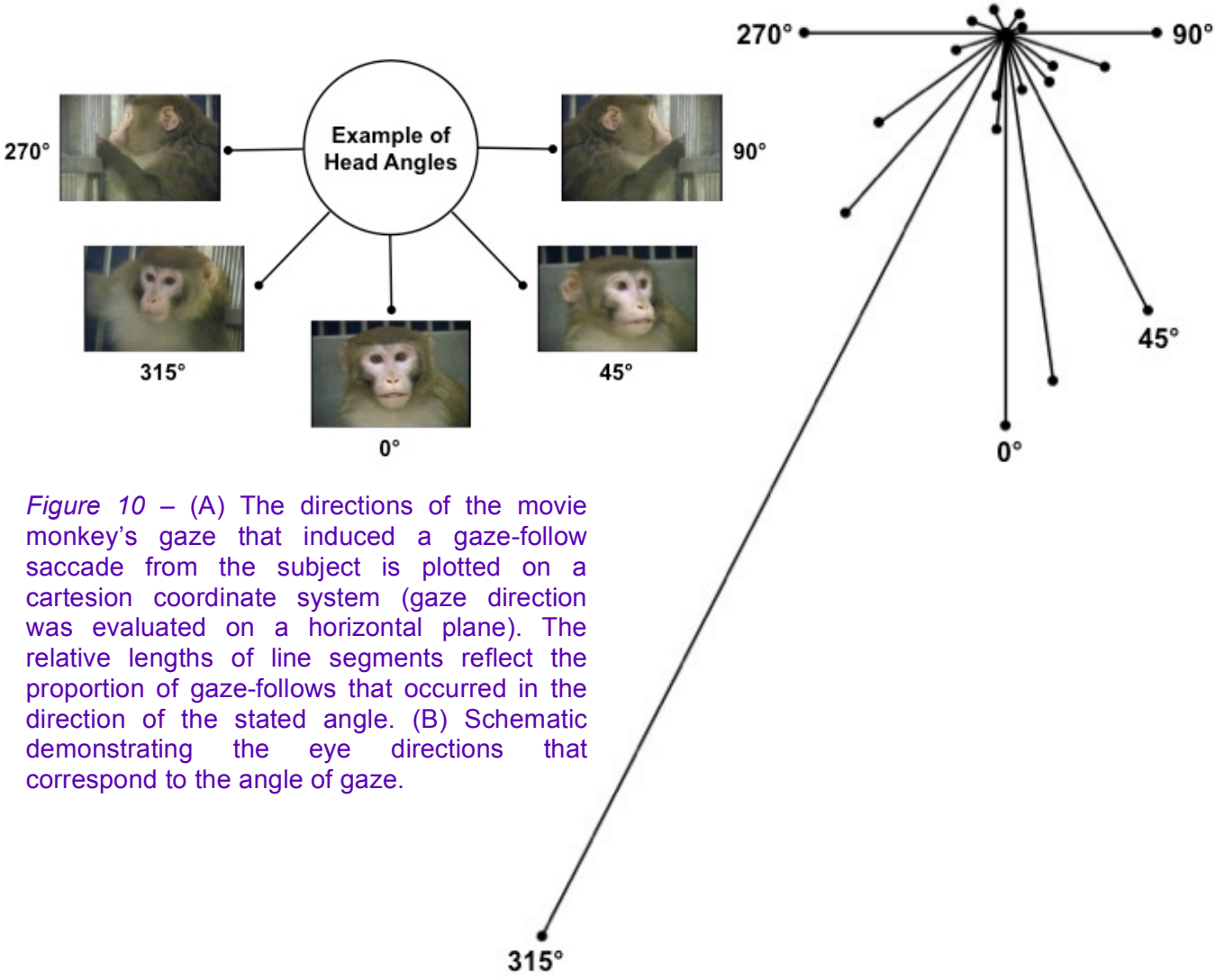

Figure 10 – (A) The directions of the movie monkey's gaze that induced a gaze-follow saccade from the subject is plotted on a cartesion coordinate system (gaze direction was evaluated on a horizontal plane). The relative lengths of line segments reflect the proportion of gaze-follows that occurred in the direction of the stated angle. (B) Schematic demonstrating the eye directions that correspond to the angle of gaze.

Scanpaths of each subject's eye movements as they viewed movies of conspecifics were scored in order to determine the effect of oxytocin on total time spent looking at the eye region of movie monkeys. Time spent looking at the eye region was split into two

distinct groups: direct eye contact (which could only occur when the movie monkey looked directly at the camera, thus establishing direct eye contact when the viewer monkey looked at the movie monkey's eyes) and averted eye looking (which occurred when the movie monkey's eyes were visible, but not looking directly at the camera). There was no effect oxytocin for either direct (Figure 11A) or averted eye looking (Figure 11B). In fact, the influence of habituation affected both the oxytocin and saline inhalation data comparably.

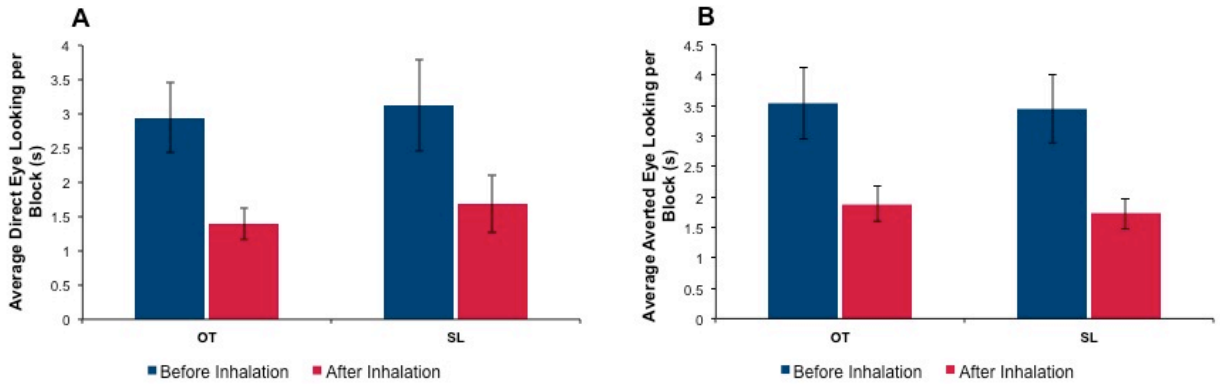

Figure 11 – (A) Oxytocin had no effect on the amount of time subjects spent making direct eye contact with movie monkeys. Both the oxytocin and saline conditions demonstrate the effect of habituation. (B) Oxytocin also had no effect on the amount of time subjects spent looking at the averted eyes of movie monkeys. Both the oxytocin and saline conditions demonstrate the effect of habituation.

Injection Experiments:

To determine the effect of oxytocin on gaze-following behavior when administered through injection, scanpaths of monkey G's eye movements as he viewed movies of conspecifics were scored for gaze-follows. The frequency of gaze-follows was averaged across blocks for each condition (oxytocin vs. aCSF) and injection location (NBM vs. amygdala) both before and after injection. On average, monkey G made relatively few gaze-follows per block (0.79 ± 0.36 on average, which is consistent with his frequency of gaze-follows in the inhalation experiment).

Monkey G did increase his frequency of gaze-following saccades after receiving microinjections of oxytocin into the NBM (Figure 12A), an effect that is not seen with injection of aCSF (Figure 12A). Habituation is reflected by the decrease in gaze-following frequency after injection of aCSF (Figure 12A). The effect, though trending toward significant, was not in fact statistically significant due to the small number of experimental sessions analyzed (n = 2). Microinjections of oxytocin into the amygdala, including the central and basolateral nuclei, did not yield any effect on gaze-following frequency (Figure 12B). In fact, habituation had a similar effect on data in both the oxytocin and aCSF injection data.

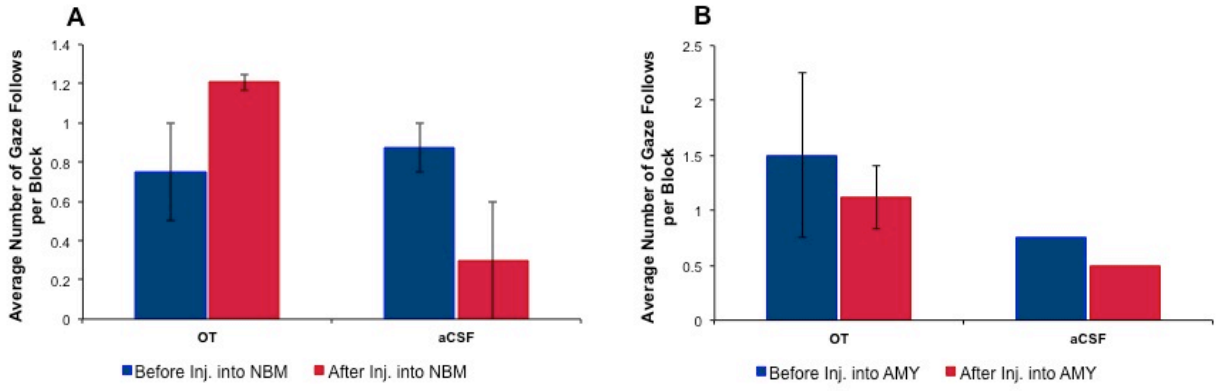

Figure 12 – (A) Oxytocin tended to increase the frequency of gaze follows when injected into the NBM, which did not occur in with injections of aCSF. Gaze following frequency was affected by habituation in the aCSF condition only. (B) Oxytocin did not have an effect on gaze-following when injected into regions of the amygdala. Gaze-following frequency in both the oxytocin and aCSF conditions were affected by habituation.

Monkey G's scanpaths were also scored in order to determine the effect of injected oxytocin on total time spent looking at the eye region of movie monkeys. There was no effect of oxytocin on either direct eye looking or averted eye looking when injected into the NBM (Figure 13A) or when injected into the amygdala (Figure 13B). In fact, the influence of habituation affected both the oxytocin and aCSF injection similarly in both locations.

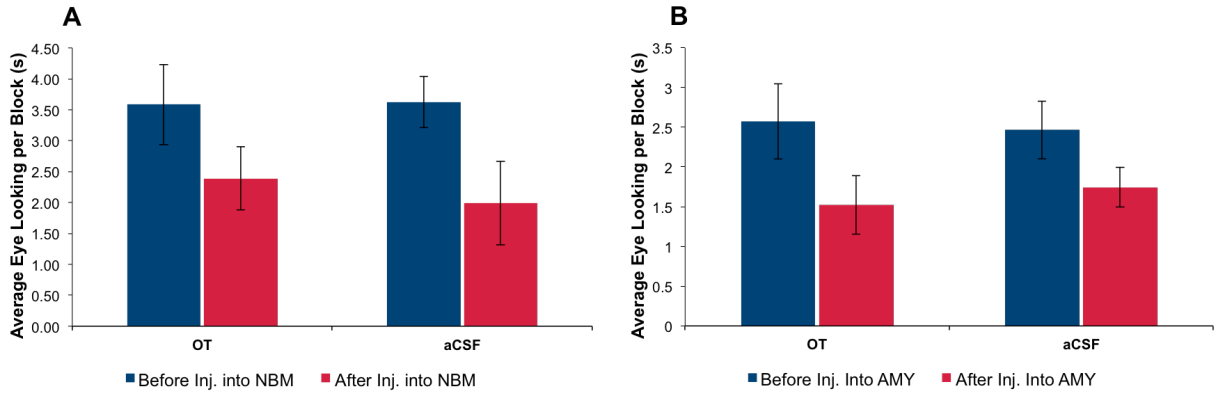

Figure 13 – Oxytocin did not have an effect on total eye looking (including direct and averted) when injected into the nucleus basalis (A) or into regions of the amygdala (B). Gaze-following frequency decreased during both conditions in both locations due to habituation.

Finally, histological analysis using Lucifer yellow fluorescence was later performed on monkey G's brain, which verified that injections were made into the intended targets in the NBM and amygdala (Figure 14).

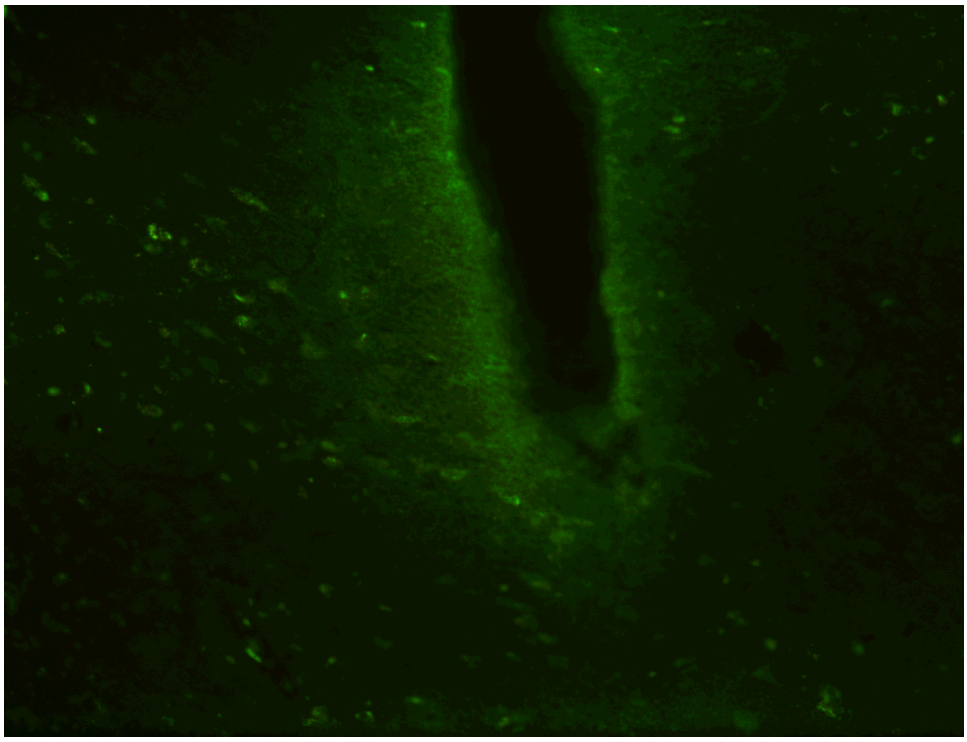

Figure 14 – Lucifer yellow fluorescence surrounding injection site centered in the NBM of the left hemisphere.

Discussion:

The main results of the experiments described here can be summarized as: (1) oxytocin overcomes habituation and increases the frequency of gaze-follows when administered through nebulization, (2) oxytocin overcomes habituation and increases the frequency of gaze-follows when injected into the NBM but not the amygdala, and (3) oxytocin has no effect on time spent looking at the eye regions of movie-monkeys when administered through nebulization or injection.

One characteristic of this experimental design is that the monkeys viewed a different set of movies each day, so movies were never repeated for both conditions. The decision to do the experiment this way was made primarily because the effect of habituation is so significant, that it is likely that there would be more gaze-follows in the first condition, regardless of whether it was oxytocin or saline. The result of this decision, however, is that the frequency of gaze-follows before inhalation of either drug is not uniform across the stimulus videos (Figures 7 and 12). This is a consequence of using different movies featuring different monkeys. Not only do movies have varying amounts of opportunities for gaze-follows to occur (based on the amount of time the movie monkey's gaze is averted), but viewer monkeys also have different preferences for certain movie monkeys, expressions (Figure 9), and gaze angles (Figure 10). Thus, the best way to interpret the data is not to compare each day, but rather compare the average difference in gaze-follow frequency before inhalation of either saline or oxytocin, and after.

When we compared the frequency of gaze-follows before and after inhalation, habituation is apparent in the saline condition (Figure 7). As previously discussed, the phenomenon of habituation is expected as subjects lose interest in repeated presentations

of the same videos. This makes the effect of oxytocin even more significant in that it overcomes habituation to the extent of increasing the frequency of gaze-follows. While this increase in gaze-follows is generally not statistically significant when compared only to the pre-oxytocin inhalation data, the increase is statistically significant relative to the decrease shown due to habituation in the control condition (inhalation of saline). However, in the case of threatening videos, inhalation of oxytocin by itself significantly increased the frequency of gaze-follows, even without taking the effects of habituation into account. This could indicate that oxytocin decreases the subject's aversion to angry faces, as previously reported in humans (Evans et al., 2010).

It is important to note that the behavioral effect of oxytocin on gaze-follows is already apparent in the first block after treatment with oxytocin, and that this effect increases in each subsequent block (Figure 8). While many recent studies have used a waiting period of approximately 45 minutes between oxytocin inhalation and the start of the behavioral task (Andari et al., 2010; Guastella et al., 2008; Monte et al., 2014; Ebitz et al., 2013), this data demonstrates that oxytocin begins having an effect as early as 10 minutes after inhalation (which is when the first post-treatment block was shown). This also means that the effect of oxytocin on increasing gaze-following behavior could be even more significant than shown in Figure 8 if given a longer wait period between inhalation and further movie presentations.

The findings from the injection experiment indicate that the NBM plays an important role in how oxytocin increases gaze-following (Figure 12A), while oxytocin injections into the central or basolateral nuclei of the amygdala have no effect (Figure 12B). Although the amygdala contains neurons that respond selectively to looking at the eyes and

to eye contact (Mosher et al., 2014), oxytocin is unlikely to interact directly with these neurons. Freeman and colleagues (2014) have shown, using quantitative neuropharmachological methods, that the monkey amygdala does not contain oxytocin receptors; the oxytocin receptors that might influence amygdala activity are located in the NBM (Figure 2). Although their methods could not establish whether the receptors were located on cholinergic neurons or other types of neurons in the NBM, the basal nucleus and accessory basal nuclei of the amygdala contain a dense network of cholinergic fibers originating in the NBM. In these nuclei, both the principal cells and the interneurons express muscarinic cholinergic receptors (e.g., Mrzljak et al., 1993, Levey et al., 1991). A recent study showed that the effect of cholinergic input from the NBM on neural activity in the amygdala depends to existent level of activity (Unal et al., 2015). When the projection neurons in the basolateral amygdala are quiescent cholinergic input from the NBM is inhibitory. On the contrary, if the projection neurons in the amygdala are already active, the cholinergic input is excitatory. These findings reinforce earlier hypotheses that the NBM sharpens the signal to noise ratio of signal processing in its targets. In this scenario, oxytocin may increase cholinergic input to the amygdala, thereby enhancing the salience of the social stimuli that activate the neurons in the amygdala. So, while our results do indicate that oxytocin in the NBM increases gaze follows, this effect could be mediated by the amygdala via cholinergic input from the NBM.

As expected, both oxytocin inhalation and injection of oxytocin into the NBM increased gaze-following frequency (Figures 7 and 12). However, there was not a similar increase in the overall time the viewer monkey spent looking at the eye region of movie monkeys (Figures 11 and 13). There are two possible explanations for this. First, while

increased eye looking is a reported effect of oxytocin in both humans and non-human primates (Andari et al., 2010; Guastella et al., 2008; Monte et al., 2014; Ebitz et al., 2013), the studies that reported these results used static images as stimuli. Since eyes are the most socially salient, and therefore very interesting, feature of static images, it is understandable that this is what viewer-monkeys focus on. When presented with more dynamic stimuli, such as movies of conspecifics, the richness of social information in these stimuli might override the tendency to allocate attentions selectively to the eyes. Perhaps, the dynamic aspects of the perceived eye movements contain sufficient information that renders prolonged staring at the eyes unnecessary. It is also possible, that the dynamic stimuli are more naturalistic and engage the social brain in scanpaths that are triggered by real-life social interaction and not by a static image that remains unchanged on the monitor. The subjects do spend a lot of time looking at the eyes of movie monkeys initially (shown in the pre-inhalation data for both conditions - Figures 11 and 13), but they tend to lose interest in the later blocks (Figures 11 and 14), which is an effect of habituation. It is possible that the subjects might forgo some eye looking in favor of focusing on other interesting and dynamic parts of the movie, which aren't present in static images.

The second possible explanation for the decrease in eye looking is simply that oxytocin increases the frequency of gaze-follows, which are saccades away from the eyes, in the direction of the movie monkey's gaze. If the viewer monkey looks away from the movie monkey's eyes to see what the movie monkey is looking at more frequently, then by definition the viewer monkey is spending less time looking at the eyes of the movie monkey.

Future Directions

While the findings of this study highlight the effects of oxytocin on gaze-following behavior in macaques, they also inspire further questions and future directions for this research. First, analyzing the movies for gaze-following opportunities (based on overall time the movie monkey's gaze is averted) and using the gaze-following data from monkeys Q, T, V (Mosher et al., 2011) and monkeys G and Z to determine if there is an overall preference to gaze-follow during movies featuring particular monkeys or expressions, can provide further insight into what type of monkeys, expressions, and behaviors induce gaze-follows. This information will allow us to modify and improve the stimulus sets for use in future experiments. Also, it is necessary to repeat the oxytocin injection experiment in additional monkeys in order to verify the effect of oxytocin in the NBM on gaze-following behavior.

To understand why our findings did not show an increase in overall time spent looking at the eyes of the stimuli (as demonstrated by Andari et al., 2010; Guastella et al., 2008; Monte et al., 2014; Ebitz et al., 2013), it is first important to see if we can replicate these findings using static stimuli. Thus, it is necessary to include static images featuring monkeys from the movie stimuli in the stimulus set for a future experiment. If eye looking is increased in the static images only then we would next want to discover why that is.

Finally, it would be fascinating to record from cells in the amygdala during inhalation experiments as this could confirm that the behavioral effects of oxytocin are mediated by the limbic system.

References:

Andari, E., J.R. Duhamel, T. Zalla, E. Herbrecht, M. Leboyer, and A. Sirigu. "Promoting Social Behavior with Oxytocin in High-functioning Autism Spectrum Disorders." Proceedings of the National Academy of Sciences 107.9 (2010): 4389-394. Web.

Anagnostou E, Soorya L, Brian J, Dupuis A, Mankad D, Smile S, Jacob S. "Intranasal Oxytocin in the Treatment of Autism Spectrum Disorders: a Review of Literature and Early Safety and Efficacy Data in Youth." Brain Research (2014). Web.

Anderson AK, Christoff K, Panitz D, De Rosa E, Gabrieli JDE. "Neural Correlates of the Automatic Processing of Threat Facial Signals." The Journal of Neuroscience 23 (2003): 5627-5633.

Auyeung B, M V Lombardo, M Heinrichs, B Chakrabarti, A Sule, JB Deakin, RA Bethlehem L Dickens, N Mooney, JA Sipple, P Thiemann, and S Baron-Cohen. "Oxytocin Increases Eye Contact During Real-time, Naturalistic Social Interaction in Males with and without Autism." Translational Psychiatry 5 (2015). Web.

Carmichael MS, Humbert R, Dixen J, Palmisano G, Greenleaf W, Davidson JM. "Plasma oxytocin increases in the human sexual response." Journal of Clinical Endocrinology & Metabolism 64 (1987): 27-31. Web.

Carter CS. (1992). Oxytocin and sexual behavior. Neurosci Biobehav Rev. 16: 131-144.

Ebitz, R. B., K. K. Watson, and M. L. Platt. "Oxytocin Blunts Social Vigilance in the Rhesus Macaque." Proceedings of the National Academy of Sciences 110.28 (2013): 11630-1635. Web.

Emery, N.j. "The Eyes Have It: The Neuroethology, Function and Evolution of Social Gaze." Neuroscience & Biobehavioral Reviews 24.6 (2000): 581-604. Web.

Evans S., Shergill S., Averbeck B. "Oxytocin Decreases Aversion to Angry Faces in an Associative Learning Task." Neuropsychopharmacology 35 (2010): 2502-2509. Web.

Floresco SB, Ghods-Sharifi S. "Amygdala – Prefrontal Cortical Circuitry Regulates Effort-Based Decision Making. Cerebral Cortex 17 (2007): 251-260. Web.

Freeman, Sara M., Inoue, Kiyoshi, Smith, Aaron L., Goodman. "The Neuroanatomical

Distribution of Oxytocin Receptor Binding and mRNA in the Male Rhesus Macaque (Macaca mulatta)." Psychoneuroendocrinology 45 (2014): 128-141. Web.

Guastella, Adam J., Philip B. Mitchell, and Mark R. Dadds. "Oxytocin Increases Gaze to the Eye Region of Human Faces." Biological Psychiatry 63.1 (2008): 3-5. Web.

Gothard KM, Battaglia FP, Erickson CA, Spitler KM, Amaral DG. "Neural Responses to Facial Expression and Face Identity in the Monkey Amygdala." Journal of Neurophysiology 97 (2007): 1671-1683.

Gothard KM, Erickson CA, Amaral DG. "How do Rhesus Monkeys (Macaca mulatta) Scan Faces in a Visual Paired Comparison Task?" Animal Cognition 7 (2004): 25-36.

Hamann SB, Ely TD, Grafton ST, Kilts CD. "Amygdala Activity Related to Enhanced Memory for Pleasant and Aversive Stimuli." Nature Neuroscience 2 (1999): 289-293. Web.

Hoffman KL, Gothard KM, Schmid MC, Logothetis NK. "Facial-expression and Gaze-selective Responses in the Monkey Amygdala." Current Biology 167 (2007): 766-772. Web.

Hughes AM, Everitt BJ, Lightman SL, Todd K. "Oxytocin in the Central Nervous System and Sexual Behavior in Male Rats." Brain Research (1987). Web.

Kendrick KM, Keverne EB. "Control of Synthesis and Release of Oxytocin in the Sheep Brain." Annals of the New York Academy of Sciences 652 (1992): 102-121. Web.

Keverne EB, Kendrick KM. "Oxytocin Facilitation of Maternal Behavior in Sheep." Annals of the New York Academy of Sciences 652 (1992): 83-101. Web.

Levey AI, Kitt CA, Simonds WF, Price DL, Brann MR. "Identification and Localization of Muscarinic Acetylcholine Receptor Proteins in Brain with Subtype-specific Antibodies." Journal of Neuroscience 11 (1991): 3218-3226. Web.

Monte, Olga Dal, Pamela L. Noble, Vincent D. Costa, and Bruno B. Averbeck. "Oxytocin Enhances Attention to the Eye Region in Rhesus Monkeys." Frontiers in Neuroscience 8 (2014): n. pag. Web.

Mosher CP, Zimmerman PE, Gothard KM. "Videos of Conspecifics Elicit Interactive Looking Patterns and Facial Expressions in Monkeys." Behavioral Neuroscience 125 (2011): 639-652.

Mosher CP, Zimmerman PE, Gothard KM. "Neurons in the Monkey Amygdala Detect

Eye Contact During Naturalistic Social Interactions." Current Biology 24 (2014): 2459-2464. Web.

Mrzljak L, Levey AI, Goldman-Rakic PS. "Association m1 and m2 Muscarinic Receptor Proteins with Asymmetric Synapses in the Primate Cerebral Cortex: Morphological Evidence for Cholinergic Modulation of Excitatory Neurotransmission." Proceedings of the National Academy of Sciences 90 (1993): 5194-5198. Web.

Muir, J.L., Page, K.J., Sirinathsignhji, D.J., Robbins, T.W., Everitt, B.J. "Excitotoxic Lesions of Basal Forebrain Cholinergic Neurons: Effects on Learning, Memory, and Attnetion." Behavioral Brain Research 57 (1993): 123-131. Web.

Peñagarikano O, Lazaro MT, Lu X, Gordon A, Dong H, Lam H, Maidment N, Murphy N, Yang XW, Golshani P, Geschwind D. "Exogenous and Evoked Oxytocin Restores Social Behavior in the *Cntnap2* Mouse Model of Autism." Science Translational Medicine 7 (2015): 271. Web.

Pedersen CA, Prange AJ Jr. "Induction of Maternal Behavior in Virgin Rats after Intracerebroventricular Administration of Oxytocin." Proceedings of the National Academy of Sciences 76 (1979): 6661-6665. Web.

Unal CT, Pare D, Zaborszky L. "Impact of Basal forebrain Cholinergic Inputs on Basolateral Amygdala Neurons." Journal of Neuroscience 35 (2015): 853-863. Web.

Whalen PJ, Davis FC, Oler JA, Kim H, Kim MJ, Neta M. "Human Amygdala Responses to Facial Expressions of Emotion." In P.J. Whalen & E.A. Phelps: The Human Amygdala: 265-288. New York: Guildford Press.

Wilensky AE, Schafe GE, Kristensen MP, LeDoux JE. "Rethinking the Fear Circuit: the Central Nucleus of the Amygdala is Required for the Acquisition, Consolidation, and Expression of Pavlovian Fear Conditioning." The Journal of Neuroscience 26 (2006): 12387-12396. Web.

Williams, Jessie R., Thomas R. Insel, Carroll R. Harbaught, and Sue C. Carter. "Oxytocin Administered Centrally Facilitates Formation of a Partner Preference in Female Prairie Voles (Microtus Ochrogasfer." Journal of Neuroendocrinology 6 (1994): 247-50. Web.

Witt DM, Carter CS, Walton DM. "Central and Peripheral Effects of Oxytocin Administration." Physiology & Behavior 37 (1990): 63-69. Web