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Award Number: W81XWH-08-1-0182

TITLE: Oxytocin and Social Support as Synergistic Inhibitors of Aversive Fear
Conditioning and Fear-Potentiated Startle in Male Rats

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REPORT DATE: May 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-05-2011		2. REPORT TYPE Final		3. DATES COVERED (From - To) 1 MAR 2008 - 30 APR 2011	
4. TITLE AND SUBTITLE Oxytocin and Social Support as Synergistic Inhibitors of Aversive Fear Conditioning and Fear-Potentiated Startle in Male Rats				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-08-1-0182	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dr. Jeff Rosen E-Mail: jrosen@udel.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Delaware Newark, DE 19716				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT We found exogenous oxytocin acts as an antianxiety agent in a fear-potentiated startle paradigm. Oxytocin given systemically (0.1 µg/kg, sc) effectively reduced background anxiety, but not specific cue-potentiated fear, when given before fear conditioning (acquisition), immediately after fear conditioning (consolidation), or before retrieval/expression of conditioned fear-potentiated startle. In contrast, oxytocin infused into the lateral ventricle only reduced background anxiety with a very large dose (20 µg). We conclude that oxytocin uniquely reduces background anxiety – an anxiety state not directly related to cue-specific fear, but sustained beyond the immediate threat. The findings also indicate that oxytocin acts as an antianxiety agent peripherally to then affect brain through indirect mechanisms. Promising initial data with a paradigm of potentiated startle after 3 weeks of social isolation have been difficult to replicate. We suggest oxytocin is promising as a drug with novel benefits for patients with PTSD.					
15. SUBJECT TERMS fear; anxiety; PTSD; startle; social isolation					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	60	19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION

PTSD can be considered a disorder of affective memory where reminiscence of aversive events becomes exaggerated, uncontrollable and frightening. Fear during PTSD also becomes generalized where it is not confined to the trauma, but occurs in other situations and stimuli too. While classic anxiolytic and antidepressant drugs have some efficacy for PTSD, they are not that efficacious in reducing this generalization of anxiety, which we call background anxiety. Newer medications via novel mechanisms are needed to target different aspect of PTSD, such as background anxiety. Exogenous oxytocin, a nonapeptide found naturally in the brain and body, may have a unique anti-anxiety effect that in addition to reducing conditioned fear it also reduces background anxiety. To test this hypothesis in an animal model of PTSD, fear-potentiated startle (FPS) in male rats was employed. FPS in rats has face-validity for PTSD because a major hallmark symptom of PTSD in humans is exaggerated startle. Using FPS, we were able to distinguish and measure different types of fear – cue-specific conditioned fear (increase in acoustic startle during trials in the presence a light previously paired with shock) and background anxiety (increase in acoustic startle during trials in the absence of the light). Interestingly, oxytocin significantly reduced background anxiety without reducing cue-specific fear. This is a novel finding that suggests oxytocin may have novel anti-anxiety properties that target unique types of anxiety particularly found in PTSD. Comparison of the route of administration of oxytocin (subcutaneous vs. intracerebroventricular) also found unanticipated results demonstrating oxytocin worked best at selectively reducing background anxiety when given peripherally as opposed to directly in brain.

BODY

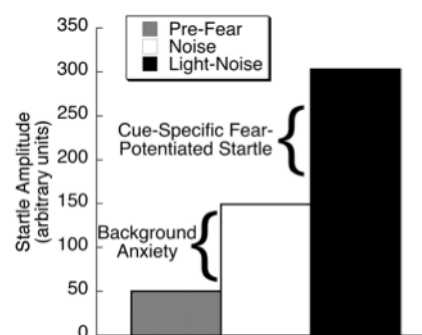
Completed work (Task 1; peripheral administration): Oxytocin Reduces Anxiety-Related Increases in Startle, But Not Cue Specific Fear-Potentiated Startle in Rats.

Publication (See Appendix 1): Missig, G., Ayers, L.W., Schulkin, J., and Rosen, J.B. (2010). Oxytocin Reduces Background anxiety in a fear-potentiated startle paradigm, *Neuropsychopharmacology*, 35:2607-2616. (doi:10.1038/npp.2010.155).

This article describes the major finding for the project. It demonstrates that peripherally administered oxytocin dose-dependently reduces background anxiety, but not cue-specific fear potentiated startle (Fig 1 for operational definitions of terms). Three experiments showed this effect on acquisition, consolidation and expression of FPS (Fig. 2 shows the selective reduction of background anxiety with oxytocin). Two additional experiments

□

Fig 1: Differences between trial types are used to define the effects on background anxiety and cue-specific fear-potentiated startle. The difference in startle between the Pre-Fear and Noise Trials is background anxiety.



demonstrated that oxytocin did not merely reduce the ability to startle and that oxytocin's effect was not due to reduction of contextually conditioned fear. We concluded oxytocin has a novel antianxiety profile that targets background anxiety – an anxiety state not directly related to cue-specific or contextual fear, but sustained beyond the immediate threat.

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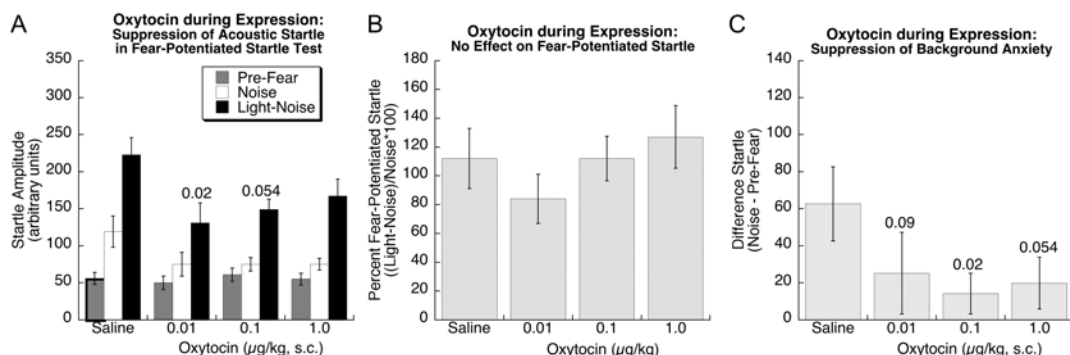


Fig. 2: Effect of oxytocin administered before the fear-potentiated startle expression test. A: Mean startle amplitudes of the three different trial types. The 0.02, and 0.054 above the Noise and Light+Noise startle scores are the respective p-values of the differences in startle between saline and the 0.01 and 0.1 μg doses of oxytocin. B: Proportional fear-potentiated startle scores. There were no statistical differences between any dose of oxytocin and saline. C: Background anxiety scores. The 0.09, 0.02, and 0.054 are the p-values of the differences in background anxiety startle scores between saline and the 0.01, 0.1, and 1.0 μg doses of oxytocin, respectively. From Missig et al., 2010; Fig. 4; see Appendix 1.

In addition to the published article, preliminary forms of this research were also presented at two meetings. The abstracts are in Appendices 2 and 3.

Completed work (Task 1, ICV administration): Oxytocin Reduces Background Anxiety in a Fear-Potentiated Startle Paradigm: Peripheral vs. Central Administration.

Manuscript in Revision (See Appendix 4): Ayers, L.W., Missig, G., Schulkin, J., and Rosen, J.B. Oxytocin Reduces Background Anxiety in a Fear-Potentiated Startle Paradigm: Peripheral vs. Central Administration, *Neuropsychopharmacology*, under review of revision.

After much experimentation, we have found effects of oxytocin given intracerebroventricularly (ICV) on reducing background anxiety. Initially we tested lower doses of oxytocin than were used for the peripheral studies because if it working in the brain effective doses should be lower. However this was not the case. Doses that worked peripherally, did not work when given ICV (Fig. 3). We therefore tried a very high dose that is 200-2000 times higher than the most effective peripherally administered doses. As shown in Fig. 4, a dose of 20 μg , ICV reduced background anxiety but appeared not to diminish cue-specific fear potentiated startle. Additional, studies found that the 20 μg ICV dose of oxytocin reduced acoustic startle when

given without prior fear conditioning (Fig. 5), indicating that this ICV dose did not selectively diminish background anxiety, but reduced the ability to startle. The data demonstrate the oxytocin given peripherally at quite low doses has robust antianxiety effects on background anxiety. The lack on selective effects with ICV injections indicates oxytocin initiates the effect in the periphery. What this peripheral site of action is and how it activates brain mechanisms will be the focus of a future grant application.

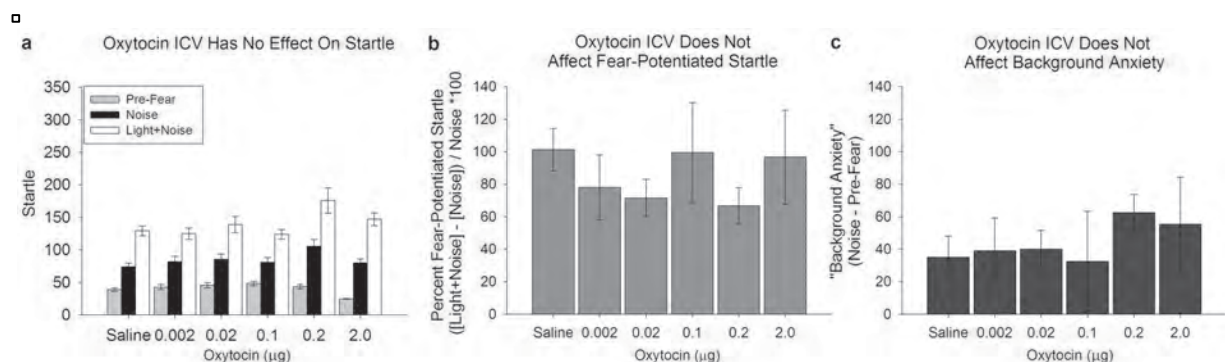


Fig 3: Oxytocin administered ICV at 5 doses 30 minutes prior to fear-potentiated startle testing. (a.) Oxytocin ICV had no effect on startle during testing. (b.) Percent fear-potentiated startle was unaffected by oxytocin. (c.) There is no effect on background anxiety at any dose of oxytocin. From manuscript in Appendix 4, Fig. 3.

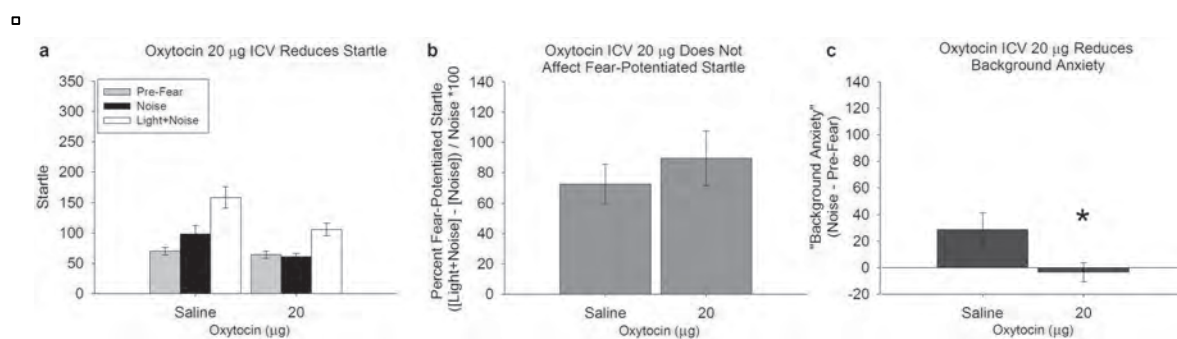


Fig. 4: Oxytocin administered ICV at a high dose (20µg) 30 minutes prior to fear-potentiated startle testing. (a.) 20µg oxytocin significantly reduced startle. (b.) Percent fear-potentiated startle was unaffected by this high dose of oxytocin. (c.) Background anxiety was significantly reduced by 20 µg oxytocin. * Indicates statistically significant from saline. From manuscript in Appendix 4, Fig. 5.

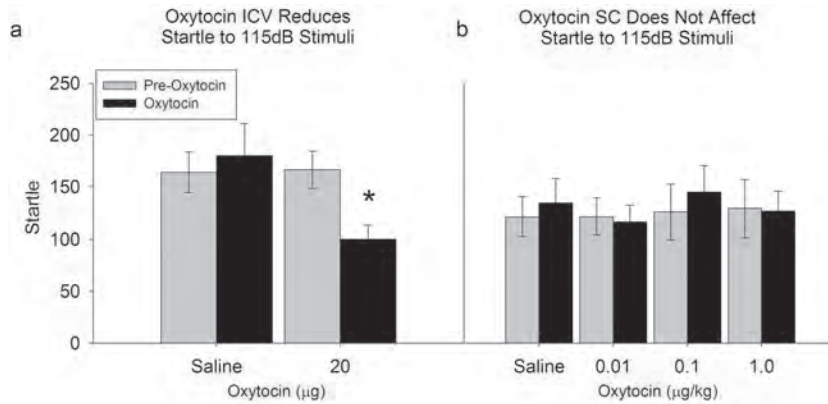
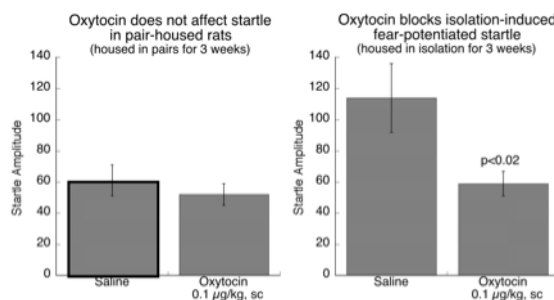


Fig. 5: Effects of oxytocin non-conditioned acoustic startle. (a.) 20 µg oxytocin administered ICV significantly decreased startle elicited by the 115 dB noise burst (*, $p < 0.009$). Startle elicited by 95 and 105 dB startle stimuli was not affected by oxytocin. (b.) By comparison, oxytocin given subcutaneously at doses effective in reducing background anxiety did not affect acoustic startle in non-conditioned rats. Startle elicited by 115 dB noise burst is shown, but startle elicited by 95 and 105 dB noise bursts was also unaffected. Data in b from Figure 5 of Missig et al., (2010). From manuscript in Appendix 4, Fig. 6.

In addition to the submitted revised manuscript (Appendix 4), an abstract of part of this work was presented at the Society for Neuroscience meeting, November 2010 (Appendix 5). An abstract of the final work will be presented at the Society for Neuroscience meeting, November, 2011.

Incomplete (Task 1; Social Support): The effects of oxytocin on buffering the effects of a lack of social support were tested. Comparison of the effects of oxytocin on rats housed in pairs versus isolated rats yielded significant differences on social isolation induced potentiation of startle. Pair-housed rats were tested for startle and then split into isolated or paired-housing for 3 weeks. Startle was tested again either with 0.1 µg/kg, sc oxytocin or saline. The results are shown in Fig. 6. While we are very excited that the finding suggests oxytocin's antianxiety effects are not confined to fear-conditioning paradigm, but extend into social buffering (and reported this in the previous annual report, we have had trouble replicating the finding. We hope to continue to refine the paradigm and accumulate robust effects sufficient for publication and preliminary findings for a future grant application.

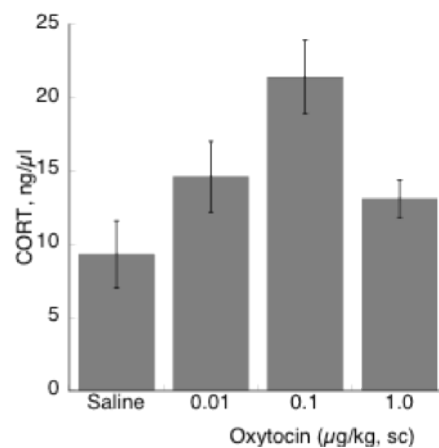
Fig. 6: Oxytocin has no effect on startle of pair-housed rats (left graph). Isolated-housing potentiates startle (right graph). Oxytocin significantly reduces the social isolation induced potentiated startle.



Incomplete (Task 2: Glucocorticoid measures): We have preliminary data on the effects of peripheral oxytocin on glucocorticoid levels in blood following the fear-potentiated startle test. 30 minutes after the fear-potentiated startle test, plasma blood was taken and the glucocorticoid levels were measured using an RIA for corticosterone (CORT). Fig. 7 shows the results. Whereas we thought oxytocin would decrease CORT because these rats displayed reduced background anxiety, the 0.1 μ g dose of oxytocin actually increased CORT levels. We do not understand the finding yet since we do not have a control group that just received oxytocin without fear conditioning and testing. We hope to pursue this interesting finding in the future, but it is unclear when.

□

Fig. 7: Levels plasma CORT 30 minutes after a fear-potentiated startle test. 0.1 μ g/kg oxytocin significantly reduced background anxiety (data not shown). Contrary to expectations, this dose significantly increased the blood levels of CORT compared to saline ($p < 0.009$).



KEY RESEARCH ACCOMPLISHMENTS

- A new psychological target for antianxiety drugs is discovered – Background anxiety is an anxiety state not directly related to cue-specific or contextual fear, but sustained beyond the immediate threat. This work has been published: Missig G, Ayers LW, Schulkin J, Rosen JB (2010). Oxytocin Reduces Background Anxiety in a Fear-Potentiated Startle Paradigm. *Neuropsychopharmacology* 35: 2607-2016. See Appendix 1.
- Systemically administered oxytocin is an effective antianxiety agent in male rats with unique properties of decreasing background anxiety but not cue-specific fear. This work has been published: Missig G, Ayers LW, Schulkin J, Rosen JB (2010). Oxytocin Reduces Background Anxiety in a Fear-Potentiated Startle Paradigm. *Neuropsychopharmacology* 35: 2607-2016. See Appendix 1.
- Intracerebroventricularly administered oxytocin only reduces acoustic startle when given in very high doses. This suggests that oxytocin is not working directly in brain. A revised manuscript of this work has been submitted to *Neuropsychopharmacology*. See Appendix 4.
- Taken together, it is concluded that peripherally administered oxytocin uniquely inhibits background anxiety, while leaving fear to a specific fear stimulus intact.
- Oxytocin potentially blocks the potentiation of startle induced by social isolation. While exciting that oxytocin might buffer the detrimental effects of social isolation, its effect has been difficult to replicate.
- The research might have implications for oxytocin as a novel therapeutic treatment for PTSD, which has a high degree of generalization of fear and anxiety.

REPORTABLE OUTCOMES

Manuscripts

1. Ayers, L.W., Missig, G., Schulkin, J. and Rosen, J.B. (revision submitted to *Neuropsychopharmacology*). Oxytocin Reduces Background Anxiety in a Fear-Potentiated Startle Paradigm: Peripheral vs. Central Administration.
2. Missig, G, Ayers, L.W., Schulkin, J. and Rosen, J.B. (2010). Oxytocin reduces background anxiety in a fear-potentiated startle paradigm. *Neuropsychopharmacology*, 35:2607-2616.

Abstracts

3. Ayers, L.W., Missig, G., Schulkin, J. and Rosen, J.B. (2011). Oxytocin administered systemically selectively reduces background anxiety, while intracerebroventricular delivery non-specifically attenuates startle. Submitted to society for Neuroscience Meeting, Washington, DC.
4. Ayers, L.W., Missig, G., Schulkin, J. and Rosen, J.B. (2010). Systemic, but not intracerebroventricular, administration of oxytocin results in an attenuation of background anxiety in fear-potentiated startle paradigm. Program No. 705.24. Society for Neuroscience: Neuroscience Meeting Planner, San Diego, CA. Online.
5. Ayers, L. W., Missig, G., Schulkin, J., Rosen, J.B. (2009). Oxytocin reduces anxiety-related increases in startle, but not cue-specific fear-potentiated startle in male rats: Relevance to PTSD. Program No. 841.17. 2009 Neuroscience Meeting Planner. Chicago, IL: Society for Neuroscience, 2009. Online.
6. Rosen, J.B., Missig, G., and Ayers, L.W. (2009). Oxytocin reduces anxiety-related increase in startle, but not cue-specific fear-potentiated startle in rats. Presented at the Military Health Research forum, Kansas City, MO, September 1. Oral presentation #S3-11 and poster presentation #P19-13.

Degrees obtained that were supported by this award

7. Galen Missig received a BS in Psychological Science, University of Delaware. He is currently a graduate student at the University of Vermont.
8. Luke Ayers received a MS in Psychology, University of Delaware. He is currently a Ph.D. candidate at the University of Delaware in the process of proposing a dissertation based upon the experience supported by this award.

Funding applied for based on work supported by this award

9. A 2nd resubmission of an NIH R01 application will be reviewed in June. The 1st submission received a 25 percentile score.

CONCLUSION

The project has been quite successful. I believe the article published in *Neuropsychopharmacology* has been received very well and will lead people to investigating background anxiety as a therapeutic target. When I presented the preliminary results at the MHRF in Kansas City in 2009 and at the Society for Neuroscience meetings in 2009 and 2010 there was a lot of interest in the work and it generated much discussion. Our more recent submitted manuscript from the data of intracerebroventricular administration of oxytocin has received enthusiastic support from the referees.

So what section

The two sets of results should produce considerable interest and possibly controversy. They suggest that oxytocin's site of direct action is not the brain, but the site of initiation of action is in the periphery to produce its actions. This may have implications for where the site of action is for the intranasal delivery of oxytocin in humans, which is thought to get into the brain but has not be definitively shown.

Appendix 1:

Missig, G, Ayers, L.W., Schulkin, J. and Rosen, J.B. (2010). Oxytocin reduces background anxiety in a fear-potentiated startle paradigm. *Neuropsychopharmacology*, 35, 2607-2616.

Oxytocin Reduces Background Anxiety in a Fear-Potentiated Startle Paradigm

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Oxytocin reportedly decreases anxious feelings in humans and may therefore have therapeutic value for anxiety disorders, such as post-traumatic stress disorder (PTSD). As PTSD patients have exaggerated startle responses, a fear-potentiated startle paradigm in rats may have face validity as an animal model to examine the efficacy of oxytocin in treating these symptoms. Oxytocin (0, 0.01, 0.1, or 1.0 µg, subcutaneously) was given either 30 min before fear conditioning, immediately after fear conditioning, or 30 min before fear-potentiated startle testing to assess its effects on acquisition, consolidation, and expression of conditioned fear, respectively. Startle both in the presence and absence of the fear-conditioned light was significantly diminished by oxytocin when administered at acquisition, consolidation, or expression. There was no specific effect of oxytocin on light fear-potentiated startle. In an additional experiment, oxytocin had no effects on acoustic startle without previous fear conditioning. Further, in a context-conditioned test, previous light-shock fear conditioning did not increase acoustic startle during testing when the fear-conditioned light was not presented. The data suggest that oxytocin did not diminish cue-specific conditioned nor contextually conditioned fear, but reduced background anxiety. This suggests that oxytocin has unique effects of decreasing background anxiety without affecting learning and memory of a specific traumatic event. Oxytocin may have antianxiety properties that are particularly germane to the hypervigilance and exaggerated startle typically seen in PTSD patients.

Neuropsychopharmacology advance online publication, 15 September 2010; doi:10.1038/npp.2010.155

Keywords: oxytocin; anxiety; fear; startle; PTSD

INTRODUCTION

Oxytocin has recently received considerable attention for its role in social behavior, and as a possible target for a number of psychiatric disorders, particularly, anxiety, post-partum depression, and autism (Carter, 2007; Heinrichs *et al*, 2009; Macdonald and Macdonald, 2010; Marazziti and Catena Dell'osso, 2008; Neumann, 2008). Oxytocin is a nonapeptide released in blood from the hypothalamo-neurohypophysial system and other peripheral organs, and in the brain within the hypothalamus, amygdala, bed nucleus of the stria terminalis, brainstem, and other regions from neurons originating in the hypothalamic paraventricular and supraoptic nuclei (Gimpl and Fahrenholz, 2001; Kiss and Mikkelsen, 2005).

Exogenous oxytocin has anxiolytic effects. Peripheral and central injections of oxytocin in rats and mice reduce anxiety in a number of tests when stress is high or induced (Rotzinger *et al*, 2010). Subcutaneous injections of oxytocin and oxytocin fragments in rats reduce retention of passive avoidance (Boccia and Baratti, 2000; de Oliveira *et al*, 2007; de Wied *et al*, 1987). Rats given low subcutaneous doses (1–4 µg/kg) of oxytocin spent more time in the center of an open field, similar to the behavior of rats given the anxiolytic benzodiazepine drug midazolam (Uvnäs-Moberg *et al*, 1994). A high-stress strain of Sprague-Dawley rats that typically perform poorly on conditioned avoidance showed significantly improved learning when given systemic oxytocin pretreatment (Uvnäs-Moberg *et al*, 2000).

In humans, exogenous intranasally administered oxytocin has anxiolytic effects in males (Domes *et al*, 2007; Heinrichs *et al*, 2003; Kirsch *et al*, 2005), diminishes aversive conditioning (Petrovic *et al*, 2008), and promotes emotional facial recognition (Di Simplicio *et al*, 2009; Fischer-Shofty *et al*, 2010) and memory (Savaskan *et al*, 2008). Oxytocin may have potential therapeutic use in social anxiety disorder (Guastella *et al*, 2009), autism (Andari *et al*,

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Received 10 May 2010; revised 8 August 2010; accepted 9 August 2010

2010), and possibly post-traumatic stress disorder (PTSD) (Pitman *et al*, 1993).

Acoustic startle as a measure of sensorimotor responsivity and anxiety (Braff *et al*, 2001; Davis *et al*, 2010; Swerdlow *et al*, 2008; Vaidyanathan *et al*, 2009) is also affected by oxytocin, but results are variable. High doses of oxytocin had no effect on startle (Feifel and Reza, 1999), but lower doses increased startle when tested in the dark phase of the day (King *et al*, 1985). Oxytocin null mice displayed low (Winslow *et al*, 2000) or normal (Caldwell *et al*, 2009) startle amplitudes. Oxytocin receptor knockout mice had normal acoustic startle (Lee *et al*, 2008). Oxytocin also did not affect pre-pulse inhibition of startle (PPI) by itself (Feifel and Reza, 1999), but disruption of PPI by phencyclidine was enhanced in oxytocin null mice (Caldwell *et al*, 2009), and oxytocin and a receptor agonist, WAY-267464, reversed the disruption in PPI induced by amphetamine and MK-801 in rats (Feifel and Reza, 1999; Ring *et al*, 2010). Highly emotional rats that have low plasma levels of oxytocin have increased startle (Uvnäs-Moberg *et al*, 1999). Similarly, Nair *et al* (2005) demonstrated that oxytocin receptor binding in the lateral septum was negatively correlated with the amplitude of startle potentiated by social isolation. Finally, humans homozygous for the G allele (GG) of a single-nucleotide polymorphism within intron 3 of the *OXTR* gene had lower levels of stress reactivity in anticipation of a startle stimulus than individuals with one or two copies of the A allele (AA and AG) polymorphism (Rodrigues *et al*, 2009). Together, these studies suggest that endogenous oxytocin and exogenously administered oxytocin modulate anxious states of rodents and humans.

Oxytocin has not been tested in fear-potentiated startle, which is often used as a measure of conditioned anticipatory anxiety and may model the hypervigilance and exaggerated startle responses typically seen in PTSD patients (Grillon and Morgan, 1999; Grillon *et al*, 2009b; Jovanovic *et al*, 2010, 2009; Morgan *et al*, 1995). One advantage of the fear-potentiated startle paradigm is that drug effects on fear or anxiety can usually be dissociated from motoric effects of drugs (Davis *et al*, 1993; Fendt *et al*, 2010; Joordens *et al*, 1998; Walker and Davis, 2002a). In the present experiments, oxytocin was administered systemically at various phases of learning, memory, and expression of fear to investigate its effects on acquisition, consolidation, and expression of conditioned fear. Our findings indicate a unique anxiolytic profile for oxytocin on startle and background anxiety, a state not directly related to cue-specific or contextually conditioned fear, but sustained beyond the immediate threat (Walker and Davis, 2002b).

MATERIALS AND METHODS

Animals

A total of 240 male Sprague–Dawley rats weighing between 225 and 250 g were obtained from Charles River Laboratories (Wilmington, MA). The rats were pair-housed in shoebox cages in a climate-controlled facility with a 0700–1900 hours light/dark cycle. Rats had free access to food and water. At 1 week after arrival, experiments were started and were performed between 0800 and 1600 hours.

All procedures were in accordance with the US National Institutes of Health Guide for the Care and Use of Experimental Animals and approved by the University of Delaware IACUC.

Apparatus

Eight identical SR Lab ventilated startle chambers with clear Plexiglas cylinders (San Diego Instruments, San Diego, CA) were used for training and testing. On one wall of each chamber, three LED lights in parallel produced 2600 lux and served as the conditioned stimulus (CS). A floor insert made of ten 4-mm diameter stainless steel tubes placed 4 mm apart inside the Plexiglas cylinder to deliver footshocks was used. Background white noise of 65 dB was continually played throughout all experimental sessions.

Experiment Design

Each experiment followed the basic paradigm: 3 days of startle acclimation/matching, 1 day of classical fear conditioning, and after a 96-h gap, a fear-potentiated startle test session. Deviations from this pattern are noted below in the Experiment sections.

Startle Acclimation/Matching

For the first 3 days of the experiment, rats were habituated to the chamber and presented with startle stimuli. For each daily session, there was a 5-min acclimation period followed by 30 trials of startle stimuli. The series of trials consisted of white noise bursts of 10 trials each of 95, 105, or 115 dB noise bursts presented in a predetermined pseudorandom pattern with a 15 s intertrial interval. On the third day of acclimation, the startle amplitudes were averaged for each rat and the mean startle score was used to sort the rats into matched groups with similar levels of startle. The rats were then rehoused and paired with a member of the same group.

Fear Conditioning

On the fourth day, the rats were classically fear conditioned to the light. Following a 5-min acclimation period, five pairings of 3 s of the light CS co-terminating with a 500 ms, 0.6 mA foot shock occurred. The intertrial intervals ranged from 60 to 180 s.

Fear-Potentiated Startle Testing

After a 96-h rest, the rats were tested for fear-potentiated startle. The testing consisted of 5 min of acclimation followed by 70 startle trials with 15 s intervals. The first 10 trials that consisted of 95 dB noise bursts were not used in any analyses. The next 60 trials consisted of 95, 105, or 115 dB noise bursts, with half presented either in the dark or co-terminating with the 3 s light CS. Thus, for each noise burst intensity, there were 10 trials in the dark and 10 trials co-terminating with the light. The trials were presented in a predetermined pseudorandom pattern.

Oxytocin Administration

Each group of rats was administered either 0, 0.01, 0.1, or 1.0 µg/ml/kg of oxytocin dissolved in saline (Bachem Americas, Torrance, CA, catalog number H-2510). The choice of doses was based on studies of de Wied *et al* (1987) and Boccia *et al* (1998). The choice of injections 30 min before the session was based on Ring *et al* (2006). A frozen stock solution of 10 µg/ml oxytocin was diluted before each experiment and maintained on ice. Injections were given subcutaneously at the scruff of the neck.

Experiment 1: Oxytocin During Acquisition

Injections were given 30 min before conditioning to examine the effect on acquisition of learned fear. Doses of 0.0, 0.01, 0.1, and 1.0 µg/kg oxytocin were tested with 12 rats in each condition for a total of 48 rats.

Experiment 2: Oxytocin During Consolidation

Injections were given 20 min after conditioning to determine the effect on fear consolidation. Again vehicle and the same three doses were tested with 12 rats in each condition.

Experiment 3: Oxytocin During Expression

Injections were given 30 min before fear-potentiated startle testing on the eighth day (96 h after acquisition) to test for the effect on expression of fear-potentiated startle. The same doses of oxytocin were tested with 12 rats per dose.

Experiment 4: Oxytocin on the Acoustic Startle Response Without Fear Conditioning

This experiment tested whether oxytocin suppressed the ability to startle. Acclimation and matching were performed similarly as previously described. On the fourth day, rats were not put into the testing chambers, nor were they conditioned (no lights, no shocks). On the eighth day, oxytocin was administered 30 min before acoustic startle testing. Instead of using a combination of Light + Noise and Noise-only trials, the 30 trials presented during acclimation was used. The same doses of oxytocin were tested with 12 rats per dose.

Experiment 5: Oxytocin on Context Fear-Potentiated Startle

In addition to fear conditioning to the explicit cue, conditioning also occurs to the context during cue-specific fear conditioning. Testing for contextually conditioned fear is typically conducted by returning the subject to the context without presentation of the explicit fear CS (Jacobs *et al*, 2010). To examine whether oxytocin influenced contextually conditioned fear-potentiated startle or not, the same 3 days of acclimation, group matching for startle response, and light-shock fear conditioning on the fourth day were performed as described above. After 96 h, rats were given saline or oxytocin, and 30 min later, instead of testing cue-specific light CS fear-potentiated startle, contextual fear was examined by presenting only Noise trials.

Thus, instead of receiving a combination of 60 Light + Noise and Noise trials, rats received 60 Noise trials in the same pseudorandom order as before. The same doses of oxytocin were tested with 12 rats per dose.

Data Analysis

For experiments 1 through 3, three startle scores were used for the statistical analyses: Pre-Fear startle, Noise, and Light + Noise. Startle amplitudes of each rat induced by the 95, 105, and 115 dB noise bursts (30 trials) from the last (third) acclimation session were averaged to obtain a single score of Pre-Fear startle. The same was done for the 30 Noise and 30 Noise + Light trials in the fear-potentiated startle test for Noise and Light + Noise scores, respectively. These scores were then used for statistical analyses.

The effect of oxytocin in the fear-potentiated startle test was analyzed by a mixed model ANOVA with a between-subject measure of dose (4 doses) and within-subject measure of fear-potentiated startle (Light + Noise vs Noise). *Post hoc* analysis of a main effect of dose on startle was performed with a Dunnett's test to compare the various doses of oxytocin to the vehicle (saline). Cue-specific conditioned fear was analyzed to two ways—using absolute fear-potentiated startle or proportional fear-potentiated startle scores. An absolute fear-potentiated startle score was computed by subtracting the average Noise startle amplitude from its average Light + Noise startle amplitude of each rat. A proportional fear-potentiated startle score for each rat was computed dividing the absolute fear-potentiated startle score by the average Noise startle amplitude. Analysis of proportional fear-potentiated startle was done to standardize the groups because fear-potentiated may be distorted by the baseline effects on oxytocin (Walker and Davis, 2002a). Dunnett's tests were used for these analyses.

A measure of change in startle amplitude after fear conditioning, which we call background anxiety, was also computed. Pre-Fear startle was compared with the Noise trials from the fear-potentiated startle test. Similar to the analysis of fear-potentiated startle described above, a mixed model ANOVA with a between-subject measure of dose and within-subject measure of background anxiety (Pre-Fear vs Noise) was performed. *Post hoc* analysis of a main effect of dose was performed with a Dunnett's test to compare the various doses of oxytocin to the vehicle (saline). A significant interaction effect was further analyzed with a Dunnett's test after the startle data was converted into background anxiety score (Noise minus Pre-Fear startle scores).

Experiments 4 and 5 did not test for Light + Noise startle. The Pre-Fear and Noise startle scores were statistically analyzed in a similar manner as the background anxiety measure of experiments 1–3. An α value of $p < 0.05$ was considered a significant difference for all the analyses described above, but trends ($p < 0.1$) are also presented in graphs.

RESULTS

The two important comparisons in this study are shown in Figure 1. Background anxiety is the comparison between Noise startle amplitude and Pre-Fear startle amplitude, and is the facilitating effect of cue-specific fear conditioning on

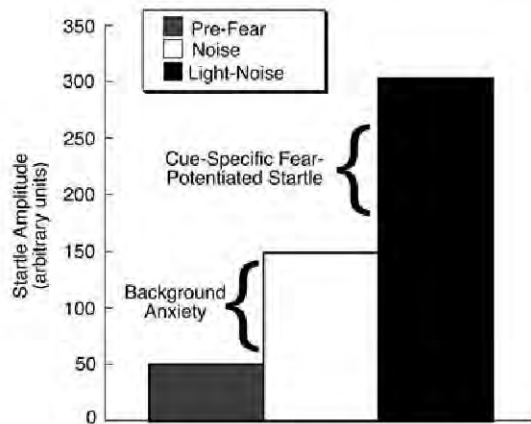


Figure 1 Sample startle responses. Startle from three different trial types were used to analyze the effects of oxytocin. Background anxiety is the increase in startle amplitude in the Noise trials during the fear-potentiated startle test compared with startle amplitude during the last acclimation session (Pre-Fear startle). Cue-specific fear-potentiated startle is the increase in startle amplitude in the Light+Noise trials compared with startle amplitude in the Noise trials during the fear-potentiated startle test.

Noise trials in the fear-potentiated startle test. Cue-specific fear-potentiated startle is the increase in Light + Noise startle amplitude compared with Noise startle amplitude due to the Light + footshock fear conditioning.

In general, regardless of when oxytocin was administered (ie, acquisition, consolidation or expression), it had similar effects on background anxiety and cue-specific fear-potentiated startle, but the effects were statistically most robust when oxytocin was administered 30 min before acquisition session or the fear-potentiated startle test. Oxytocin dose dependently diminished background anxiety and acoustic startle both in the presence and absence of light, but had no specific effect on cue-specific fear-potentiated startle.

Experiment 1: Oxytocin Effects on Acquisition

There was a significant main effect of cue-specific fear-potentiated startle (Light + Noise trials different from Noise trials, $F_{1,44} = 106.1$, $p < 0.0001$) and a trend for a main effect of oxytocin dose on startle amplitude ($F_{3,44} = 2.33$, $p < 0.088$). A Dunnett's test revealed a significant reduction in acoustic startle by 0.1 μ g oxytocin compared with saline ($p < 0.034$, Figure 2a). There was no interaction effect indicating that oxytocin did not affect cue-specific fear-potentiated startle using absolute fear-potentiated startle scores. This was supported using proportional fear-potentiated startle scores (Figure 2b). Background anxiety was only marginally reduced by oxytocin. A mixed model ANOVA revealed a main effect of an increase in startle in Noise trials compared with Pre-Fear trials ($F_{1,44} = 27.0$, $p < 0.0001$). A Dunnett's test showed that there was a trend for the 0.1 μ g dose of oxytocin to diminish background anxiety compared with saline ($p = 0.064$, Figure 2c).

Experiment 2: Oxytocin Effects on Consolidation

Similar to oxytocin given before acquisition, there was a significant within-measure main effect of fear-potentiated startle ($F_{1,44} = 147.8$, $p < 0.0001$; Figure 3a). There was a

trend for a between-measure main effect of oxytocin on startle amplitude ($F_{3,44} = 2.81$, $p < 0.092$) and a Dunnett's test suggests this is because of reduced startle with 0.1 μ g oxytocin compared with saline ($p < 0.046$, Figure 3a). There was a significant interaction effect ($F_{3,44} = 3.06$, $p < 0.038$) suggesting an effect of oxytocin on cue-specific fear-potentiated startle using absolute fear-potentiated startle scores. However, a Dunnett's test using proportional fear-potentiated startle scores was not significant indicating oxytocin did not affect cue-specific fear-potentiated startle when the scores were standardized (Figure 3b). Testing for significance of background anxiety, there was a significant overall increase in Noise startle ($F_{1,44} = 173.2$, $p < 0.0001$), but no main effect of oxytocin dose on startle amplitude, nor an interaction. A Dunnett's test suggests there was a trend for a reduction in background anxiety with 0.1 μ g oxytocin ($p < 0.08$).

Experiment 3: Oxytocin Effects on Expression

Scores were not obtained from one rat because of equipment malfunction. The effects of oxytocin given 30 min before the fear-potentiated startle test were similar to the effects on acquisition and consolidation. There was a significant main effect of fear-potentiated startle ($F_{1,43} = 129.18$, $p < 0.0001$) and a significant main effect of oxytocin dose on startle amplitude ($F_{3,43} = 3.07$, $p = 0.038$). Shown in Figure 4a, Dunnett's test revealed that the 0.01 μ g dose of oxytocin significantly diminished startle ($p = 0.022$) and the 0.1 μ g dose just missed significantly reducing startle ($p = 0.054$). There was no effect of oxytocin on fear-potentiated startle using either absolute or proportional scores of fear-potentiated startle (Figure 4b). Analyzing background anxiety, there was an overall increase in startle to Noise compared with Pre-Fear startle ($F_{1,43} = 23.93$, $p < 0.0001$). Oxytocin reduced background anxiety (Figure 4c). There was no main effect of oxytocin dose on startle amplitude, but there was significant interaction ($F_{3,43} = 3.14$, $p = 0.035$). A Dunnett's test on the interaction effect revealed that 0.1 μ g oxytocin significantly reduced background anxiety compared with saline ($p = 0.022$), and the other two oxytocin doses displayed a trend for reducing background anxiety (0.001 μ g, $p = 0.094$; 1.0 μ g, $p = 0.054$).

Experiment 4: Oxytocin does not Reduce the Ability to Startle

The previous experiments demonstrated that oxytocin reduces acoustic startle both in the absence and presence of the fear conditioned stimulus. While we are calling this a reduction in background anxiety, an alternative explanation is that oxytocin simply interferes with the ability to startle or respond to the acoustic stimulus. To test whether oxytocin is merely reducing the startle response, rats were not fear conditioned, but tested for startle amplitude with or without oxytocin. Within-subject comparisons were made between startle before receiving oxytocin and 30 min after oxytocin administration (Figure 5a). There were no effects of any dose of oxytocin on startle amplitude. Thus, oxytocin may not merely reduce the ability to startle, but seems to reduce startle subsequent to fear conditioning.

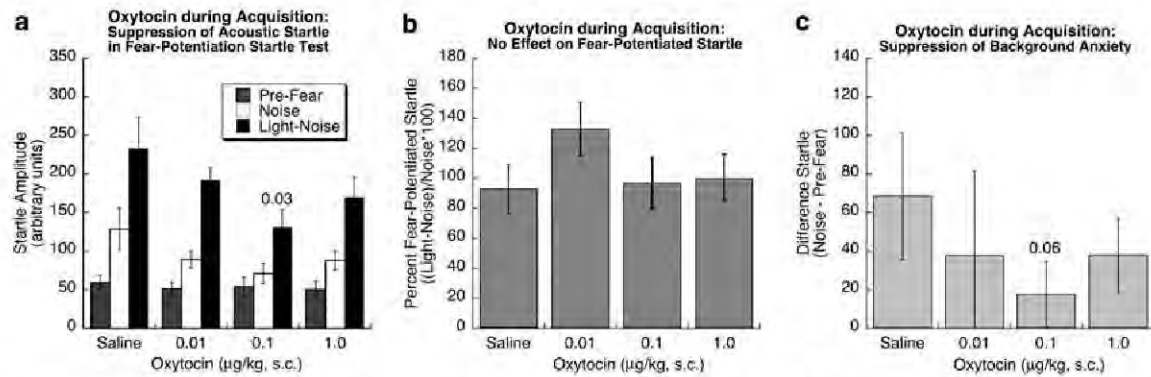


Figure 2 Effect of oxytocin administered before the acquisition phase. (a) Mean startle amplitudes of the three different trial types. The 0.03 above the Noise and Light + Noise startle scores is the p -value of the difference in startle between saline and 0.1 μg oxytocin. No other comparisons approached statistical significance. (b) Proportional fear-potentiated startle scores. There were no statistical differences between any dose of oxytocin and saline. (c) Background anxiety scores. The 0.06 is the p -value of the difference in background anxiety startle scores between saline and 0.1 μg oxytocin. No other comparisons approached statistical significance.

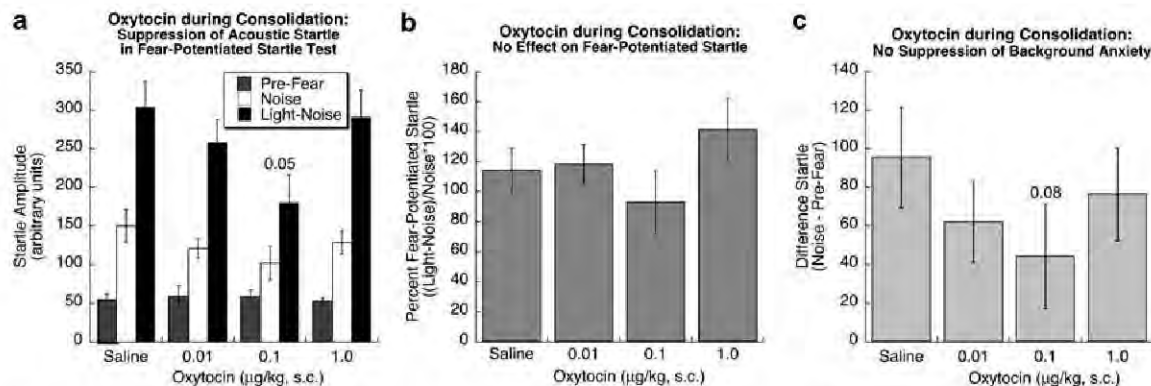


Figure 3 Effect of oxytocin administered in the consolidation phase. (a) Mean startle amplitudes of the three different trial types. The 0.05 above the Noise and Light + Noise startle scores is the p -value of the difference in startle between saline and 0.1 μg oxytocin. No other comparisons approached statistical significance. (b) Proportional fear-potentiated startle scores. There were no statistical differences between any dose of oxytocin and saline. (c) Background anxiety scores. There were no statistical differences between any dose of oxytocin and saline, but the 0.1 μg dose approached significance ($p < 0.08$).

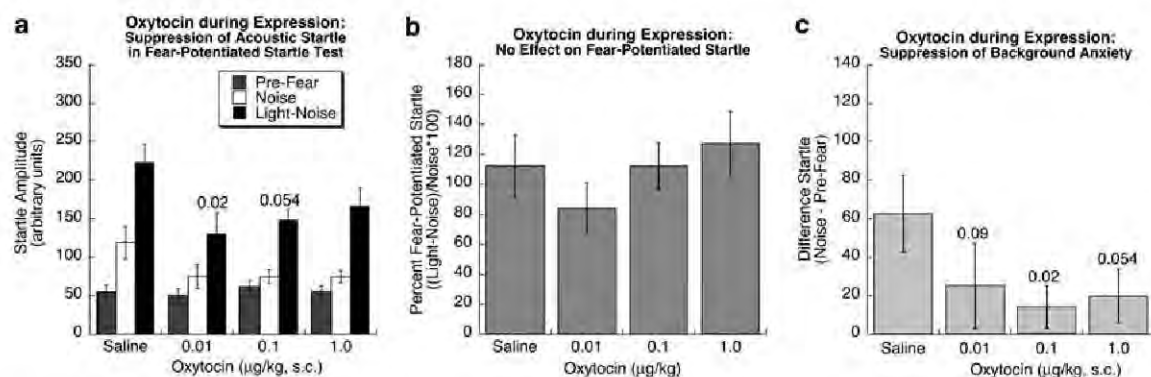


Figure 4 Effect of oxytocin administered before the fear-potentiated startle expression test. (a) Mean startle amplitudes of the three different trial types. The 0.02, and 0.054 above the Noise and Light + Noise startle scores are the respective p -values of the differences in startle between saline and the 0.01 and 0.1 μg doses of oxytocin. (b) Proportional fear-potentiated startle scores. There were no statistical differences between any dose of oxytocin and saline. (c) Background anxiety scores. The 0.09, 0.02, and 0.054 are the p -values of the differences in background anxiety startle scores between saline and the 0.01, 0.1, and 1.0 μg doses of oxytocin, respectively.

It is possible, however, that the lack of an effect of oxytocin on startle amplitude was because startle levels were very low in this experiment, and oxytocin may be more

effective in reducing high levels of startle like those generated in experiments 1 through 3 following fear conditioning. We therefore reanalyzed the data of experiment 4

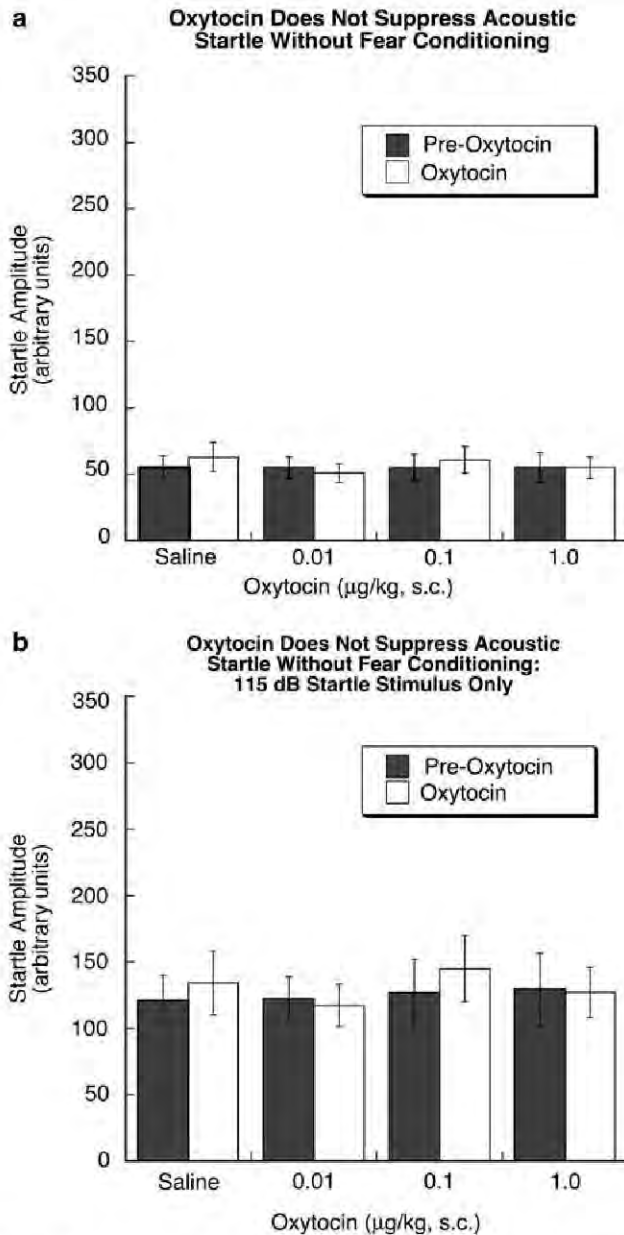


Figure 5 No effect of oxytocin on acoustic startle in rats that were not fear conditioned. (a) Startle amplitudes averaged from the 95, 105, and 115 dB startle stimuli. (b) Startle amplitudes from the 115 dB startle stimulus only.

using startle amplitudes induced by the three startle stimulus intensities, 95, 105, and 115 dB noise bursts, individually. There were no effects of oxytocin on startle elicited at any of these intensities. The mean pre-oxytocin and oxytocin startle amplitudes of the saline group induced by the 115 dB noise burst were 134 and 145, respectively (Figure 5b). These amplitudes are similar to the mean of the combined 95, 105, and 115 dB induced startle amplitudes of the Noise trials in the saline groups after fear conditioning in experiments 1 through 3, in which the startle amplitude means ranged from 119 to 150 startle units. Therefore, because similar levels of startle amplitude were reduced by oxytocin following fear conditioning, but not affected by oxytocin without previous fear conditioning, it is likely that

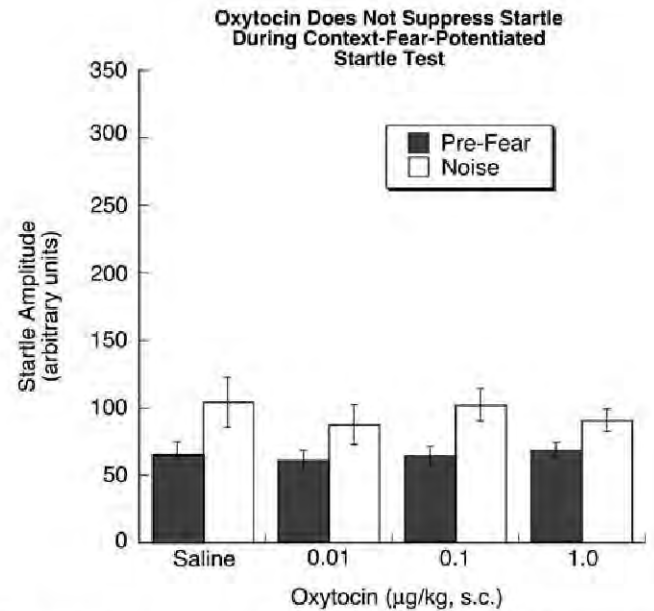


Figure 6 Test of contextually conditioned fear. No effect of oxytocin on acoustic startle in fear-conditioned rats not presented with the light CS during startle testing. There was a significant increase in startle in the Noise test compared with the Pre-Fear test in all the groups, indicating contextually conditioned fear. There were no differences in startle between the saline and oxytocin groups.

oxytocin reduces background anxiety and not the ability to startle.

Experiment 5: Oxytocin does not Reduce Contextually Conditioned Fear

Whereas we suggest that oxytocin is reducing background anxiety, it is possible that oxytocin interferes with conditioned contextual fear instead. To test this explanation, rats were tested for Pre-Fear acoustic startle amplitude, fear conditioned to the light, but then tested for startle without presenting the fear-conditioned light. Thus, if oxytocin decreased startle in the test without ever presenting the fear CS, it would indicate that oxytocin reduced contextually conditioned fear. There was a significant main effect of an increase in startle after fear conditioning ($F_{1,44} = 47.62$, $p < 0.0001$), but no significant main effect of oxytocin at any dose, nor an interaction effect (Figure 6). The results indicate that contextually conditioned fear was produced, but oxytocin did not reduce this conditioned fear as measured by startle amplitude, and suggest that the effects of oxytocin on startle in experiments 1 through 3 were due to its effects on some kind of background anxiety that is different from contextually conditioned fear.

DISCUSSION

The results of the present experiments indicate that oxytocin has unique effects on startle as measured in a fear-potentiated startle paradigm. Oxytocin did not have specific effects on cue-specific conditioned fear-potentiated startle, which is different from the cue-specific reduction of

fear-potentiated in rodents and monkeys by antianxiety drugs such as diazepam and buspirone (Davis, 1979; Joordens *et al*, 1998; Kehne *et al*, 1988; Risbrough *et al*, 2003; Winslow *et al*, 2007). Oxytocin, however, had a novel suppressant effect on startle, both in the presence and absence of the fear CS, but only if the fear CS was presented during the test. Furthermore, the increase in startle to Noise alone subsequent to fear conditioning (ie, background anxiety) was diminished by oxytocin. This unusual effect suggests that exogenous oxytocin acts as an anxiolytic agent, but does not diminish learned fear to a cue-specific or context CS. As discussed later, oxytocin may have particular therapeutic relevance for PTSD patients.

Subcutaneous oxytocin was shown to reduce acoustic startle given either during acquisition, consolidation, or expression of conditioned fear. Cue-specific fear-potentiated startle was not affected by oxytocin given at acquisition or expression (and with the proportional, but not absolute, fear-potentiated startle measure for consolidation), indicating that even though oxytocin diminished startle, there was no effect of oxytocin on cue-specific fear in the learning and expression phases. It is possible that oxytocin given during acquisition blunted nociception (Lundeberg *et al*, 1994) during fear conditioning, but there was no evidence of reduced cue-specific fear-potentiated startle in the acquisition experiment. Oxytocin also did not reduce the expression of acoustic startle in nonconditioned rats, nor contextually conditioned fear. These experiments indicate that oxytocin did not interfere with the ability to startle, nor the ability to learn cue-specific and contextually conditioned fear.

It is possible that oxytocin given before the fear-potentiated startle test reduced cue-specific fear. In this explanation, fear activated by the fear-conditioned light lingers through the 15-s intertrial intervals to enhance startle in the Noise-alone trials. Oxytocin might reduce cue-specific fear and consequently suppress the lingering fear throughout the intertrial interval and the Noise-alone trials. Although this possibility was not tested directly, de Jongh *et al* (2003) demonstrated that startle was not potentiated when the Noise was delivered 1–5 s after the offset of the light CS. This suggests that in our experiments the increase of startle in Noise-alone trials and its reduction by oxytocin were not due to cue-specific fear persisting into the Noise trials. Nonetheless, this explanation would need to be tested empirically before it is firmly rejected, possibly by testing whether there are lingering effects of the cue-specific fear CS in a novel context.

We hypothesize that oxytocin diminishes what we call background anxiety. This is an anxiety state not directly related to the cue-specific fear CS nor contextually conditioned fear cues, but is activated by the fear CS. This background state is evident during the testing of fear-potentiated startle by an increase in acoustic startle during Noise trials compared with acoustic startle in the Pre-Fear startle tests. Startle both in the absence and presence of the light fear CS was suppressed by oxytocin, but only if the light fear CS was presented during the fear-potentiated startle test session. Oxytocin given before acquisition or during consolidation could also diminish background anxiety without affecting learning and memory. The reduced background anxiety would then carryover to

the test of expression to diminish startle when exogenous oxytocin was not present. Thus, oxytocin might be uniquely effective in reducing some type of background anxiety during a threatening situation that is not cue-specifically nor context-specifically conditioned.

A background anxiety-like phenomenon in a fear-potentiated startle paradigm has been observed before. Concomitant with intra-amygdala NMDA receptor blockade of cue-specific fear-potentiated startle, Walker and Davis (2002b) found a persistent increase in 'baseline' startle in both Noise and Light + Noise trials coinciding with the first light fear CS presentation. Background anxiety appears to be activated by cue-specific fear, but might be independent of it, likely because the two phenomena are subserved by different neural circuits (Walker and Davis, 2002b).

We conducted a test for contextually conditioned fear typically used in fear-conditioning experiments (Jacobs *et al*, 2010). Oxytocin had no effect on the contextual fear-conditioned increase in startle, which is different from the reduction of contextually conditioned fear in CRH receptor knockout mice using a shock-potentiated startle paradigm (Risbrough *et al*, 2009). Shock-potentiated startle, in which no explicit cues are paired with shock (Davis, 1989), enhances startle when wild-type animals are returned to the shock chamber (McNish *et al*, 1997; Richardson, 2000; Risbrough *et al*, 2009). Antagonism or knockout of CRH receptors reduces contextually conditioned shock-potentiated startle, but cue-specific fear-potentiated startle is not affected (Risbrough *et al*, 2009). Our conditioning protocol of contextual fear was different from the shock-potentiated startle paradigm, in that shock was paired with an explicit cue, relegating context conditioning to the background. In shock-potentiated startle, there is no cue-specific stimulus, and thus the context acts as a foreground stimulus similar to an explicit cue (Rescorla and Wagner, 1972). Whether oxytocin is also ineffective in a shock-potentiated startle paradigm with context as a foreground cue is a question for further research.

In our paradigm, oxytocin was effective at very low doses in the submicrogram range. Most studies of peripheral injections of oxytocin on anxiety tests (eg, elevated plus maze, light-dark box, open field, and acoustic startle) test doses in the milligram range (Feifel and Reza, 1999; King *et al*, 1985; Rotzinger *et al*, 2010). The submicrogram range effective in our studies is similar to those used in many intracerebroventricular and intracerebral infusion studies (Rotzinger *et al*, 2010). However, our doses are similar to those used in studies of peripherally administered oxytocin on inhibitory avoidance in rats (de Oliveira *et al*, 2007; de Wied *et al*, 1987; Kovacs *et al*, 1978) and post-training administration in mice (Boccia *et al*, 1998). Thus, startle appears to be as sensitive behavioral measure as passive avoidance for peripherally administered oxytocin, but does not answer the question of whether the site(s) of action are peripheral or central. Peripheral and central oxytocin systems are regulated differently, release very different amounts of oxytocin, and metabolize oxytocin at different rates, suggesting that the two systems are largely independent (Veening *et al*, 2010). We have preliminary data that oxytocin infused into the lateral ventricle in the same range of doses we administered subcutaneously might not reduce fear-potentiated startle or background anxiety (Ayers *et al*,

2010). In the periphery, oxytocin might possibly be acting by modulating glucocorticoid release at the adrenal glands (de Oliveira *et al*, 2007) or at the heart and vasculature to influence heart rate and blood pressure, as oxytocin receptors are located in these organs (Kiss and Mikkelsen, 2005). Clearly, much more research is needed before the sites of action and mechanisms of oxytocin on background anxiety are known.

The unique effects of oxytocin on startle in the fear-potentiated startle paradigm may have particular relevance for PTSD. Potentiation of startle in PTSD patients may be particularly sensitive to 'context fear' or 'contextualization' (Grillon, 2002; Liberzon and Sripada, 2008; Rougemont-Bücking *et al*, 2010), but not cued fear. The nature of this context fear in human studies is not clear—it may be a result of contextual fear conditioning, verbal instructions of the experiment, or increased fear/anxiety induced by the aversiveness of the experiments (Böcker *et al*, 2001, 2004; Grillon, 2002; Rougemont-Bücking *et al*, 2010). Context fear might be the same as what we call background anxiety, that is, 'fear-potentiated startle is riding on an already elevated baseline' (Grillon, 2002). In our case, the background anxiety is not contextually conditioned fear, and is likely analogous to the hypervigilance and sensitized emotional anticipation (Rosen and Schulkin, 1998) hypothesized to increase startle in the face of perceived threats accompanying patients with PTSD and panic disorder (Grillon *et al*, 1994; Grillon and Morgan, 1999; Grillon *et al*, 2009b; Morgan *et al*, 1995). In this regard, combat veterans with PTSD also display disruptions in PPI (Grillon *et al*, 1998, 1996), a nonlearned measure of sensorimotor gating (Braff *et al*, 2001), and oxytocin and an oxytocin receptor agonist reverse drug-induced disruption in PPI in rodents (Feifel and Reza, 1999; Ring *et al*, 2010). Therefore, oxytocin might specifically alleviate one or more physiopathologies of PTSD.

The effect of oxytocin on background anxiety in our fear-potentiated startle studies in rats is also reminiscent of the findings from some studies with anxiolytic and antidepressant drugs on context fear in humans, in which aprazolam, diazepam, oxazepam, and a 2-week treatment of citalopram reduce increased baseline startle, but not cue-specific fear-potentiated startle (Baas *et al*, 2002; Grillon *et al*, 2006, 2009a). This does not appear to be due to sedative effects of the drugs, but to a reduction in context fear (Grillon *et al*, 2006). Oxytocin similarly reduces increased background anxiety without diminishing cue-specific fear-potentiated startle, and does not appear to produce sedation, or at least, diminish the ability to startle. Testing of oxytocin in fear-potentiated startle in humans awaits future research.

ACKNOWLEDGEMENTS

This work was supported by Grant W81XWH-08-1-0182 from the Congressionally Directed Medical Research Programs, US Army Medical Research and Materiel Command.

DISCLOSURE

The authors declare no conflict of interest.

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Appendix 2:

2009 MHRF Oral presentation #S3-11 and poster presentation #P19-13

OXYTOCIN REDUCES ANXIETY-RELATED INCREASES IN STARTLE, BUT NOT CUE-SPECIFIC FEAR-POTENTIATED STARTLE IN RATS

Jeffrey B. Rosen, Galen Missig, and Luke W. Ayers

Oxytocin increases trustworthiness and well-being, while decreasing anxious feelings in men and women. Oxytocin, therefore, may have therapeutic value for anxiety disorders, like post-traumatic stress disorder (PTSD). To test this hypothesis, the effects of oxytocin were assessed on fear-potentiated startle in male rats. Because PTSD patients have exaggerated startle responses, fear-potentiated startle in rats has face validity as an animal model to examine the effects of oxytocin on fear-exaggerated startle.

Methods: Fear-potentiated startle male Sprague-Dawley rats (225–250 g) from Charles River were housed in pairs. Startle was measured in a startle-sensitive apparatus. There were three phases of the fear-potentiated startle paradigm. Rats were first given a series of acoustic startle stimuli (95, 105, and 115 dB 50 ms white noise) on three consecutive days to determine their baseline startle amplitude. They then received Pavlovian fear conditioning of five pairings of a 3 s light co-terminating with a 500 ms, 0.6 mA footshock. Four days later, rats were tested for long-term memory of conditioned fear by delivering startle stimuli either in the presence or absence of the fear conditioned light. Fear-potentiated startle was defined as higher amplitude startle in the presence of the light compared to startle in its absence. Oxytocin (0, 0.01, 0.1, or 1.0 µg, s.c.) was administered 30 minutes before either fear conditioning, immediately after fear conditioning, or before fear-potentiated startle testing to assess its effects on acquisition, consolidation, and expression of conditioned fear, respectively. Startle amplitude without fear conditioning. The effects of oxytocin also were assessed on acoustic startle without fear conditioning. Rats were given a random series of acoustic startle stimuli on three consecutive days to determine their baseline startle amplitude. Four days later rats received 0, 0.01, 0.1, or 1.0 µg, s.c. oxytocin 30 minutes before another series of acoustic startle stimuli. Differences in startle amplitude before oxytocin and during oxytocin were analyzed.

Results: Oxytocin had similar dose-dependent effects on startle during the fear-potentiated startle test when administered at any of the three phases (acquisition, consolidation, or fear expression). There were no specific effects on fear-potentiated startle. However, startle both in the presence and absence of the light was diminished by 0.1 µg of oxytocin, regardless of when oxytocin was administered. This indicated that acoustic startle, but not fear-potentiated startle, was diminished by oxytocin. To examine whether oxytocin interacted with fear conditioning, oxytocin was tested on startle of rats without prior fear conditioning. There was no effect of oxytocin at any of the doses tested.

Conclusions and Impact: Peripheral administration of oxytocin did not diminish cue-specific conditioned fear, but reduced nonspecific anxiety. The findings suggest oxytocin has unique effects of decreasing generalized anxiety without affecting learning and memory of a specific traumatic event. Oxytocin may have anti-anxiety properties that are particularly germane to the generalization of trauma typically seen in PTSD patients.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-08-1-0182.

Appendix 3:

2009 Society for Neuroscience Abstract # 841.17

Oxytocin reduces anxiety-related increases in startle, but not cue-specific fear-potentiated startle in male rats: Relevance to PTSD.

Luke W. Ayers, Galen Missig, Jay Schulkin and Jeffrey B. Rosen

Oxytocin reportedly decreases anxious feelings in humans and may therefore have therapeutic value for anxiety disorders, like post-traumatic stress disorder (PTSD). Since PTSD patients have exaggerated startle responses, a fear-potentiated startle paradigm in rats may have face validity as an animal model to examine the efficacy of oxytocin in treating these symptoms. Male Sprague-Dawley rats were used in a 3-phase fear-potentiated startle paradigm. Rats were first given a series of acoustic startle stimuli (95, 105 and 115 dB, 50 ms duration) on 3 consecutive days to determine baseline startle amplitude. They then received Pavlovian fear conditioning of five pairings of a 3 s light co-terminating with a 500 ms, 0.6mA footshock. Four days later, rats were tested for conditioned fear by delivering startle stimuli either in the presence or absence of the fear conditioned light. Fear-potentiated startle was defined as higher amplitude startle in the presence of the light compared to startle in its absence. Oxytocin (0, 0.01, 0.1, or 1.0 µg, s.c.) was given 30 min before fear conditioning, immediately after fear conditioning, or 30 min before fear-potentiated startle testing to assess its effects on acquisition, consolidation and expression of conditioned fear, respectively. Startle both in the presence and absence of the light was significantly diminished by oxytocin (0.1 µg/kg) when administered at any of the three phases (acquisition, consolidation, or fear expression). There was no specific effect on fear-potentiated startle. Oxytocin also had no effects on acoustic startle during testing without previous fear conditioning. Further, in a context-conditioned test, previous light-shock fear conditioning did not increase acoustic startle during testing when the light was not presented. The data suggest that oxytocin did not diminish cue-specific conditioned fear, nor contextual fear, but reduced nonspecific anxiety. This suggests that oxytocin has unique effects of decreasing generalized anxiety without affecting learning and memory of a specific traumatic event. Oxytocin may have antianxiety properties that are particularly germane to the generalized hypervigilance and exaggerated startle typically seen in PTSD patients.

Supported by: Department of Defense Post-Traumatic Stress Disorder/Traumatic Brain Injury Research Program of the Office of the Congressionally Directed Medical Research Programs

Appendix 4:

Revised manuscript submitted for review to *Neuropsychopharmacology*.

Oxytocin Reduces Background Anxiety in a Fear-Potentiated Startle Paradigm: Peripheral vs.
Central Administration

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Running title: oxytocin and fear-potentiated startle

Abstract

Oxytocin is known to have anti-anxiety and anti-stress effects. Using a fear-potentiated startle paradigm in rats, we previously demonstrated that subcutaneously administered oxytocin suppressed acoustic startle following fear conditioning compared to startle before fear conditioning (termed background anxiety), but did not have an effect on cue-specific fear-potentiated startle. The findings suggest oxytocin reduces background anxiety, an anxious state not directly related to cue-specific fear, but sustained beyond the immediate threat. The goal of the present study was to compare the effects of centrally and peripherally administered oxytocin on background anxiety and cue-specific fear. Male rats were given oxytocin either subcutaneously (SC) or intracerebroventricularly (ICV) into the lateral ventricles before fear-potentiated startle testing. Oxytocin doses of 0.01 and 0.1 $\mu\text{g/kg}$ SC reduced background anxiety. ICV administration of oxytocin at doses from 0.002 to 20 μg oxytocin had no effect on background anxiety or cue-specific fear-potentiated startle. The 20 μg ICV doses of oxytocin did reduce acoustic startle in non-fear conditioned rats. These studies indicate that oxytocin is potent and effective in reducing background anxiety when delivered peripherally, but not when delivered into the cerebroventricular system. Oxytocin given systemically may have antianxiety properties that are particularly germane to the hypervigilance and exaggerated startle typically seen in many anxiety and mental health disorder patients.

Keywords: oxytocin, anxiety, fear, startle, PTSD

Introduction

Oxytocin is nonapeptide commonly known for its role in functions such as childbirth, breast feeding, pair-bonding, social behaviors, feeding, drinking, and the stress response (Carter *et al*, 2008; Gimpl and Fahrenholz, 2001; Neumann, 2008). This peptide is produced both peripherally at the uterus, ovaries, corpus luteum, prostate gland, testis, kidneys, adrenal gland and heart and in the central nervous system in neurons of the paraventricular and supraoptic nuclei of the hypothalamus (Gimpl *et al*, 2001). A large population of these hypothalamic cells project to the posterior pituitary gland where the peptide is released into the blood. A smaller population of hypothalamic neurons also projects directly to the thalamus, hypothalamus, septum, striatum, hippocampal formation, olfactory bulbs, amygdala, bed nucleus of the stria terminalis, and numerous brainstem nuclei (Gimpl *et al*, 2001; Kiss and Mikkelsen, 2005). It is thought that these central projections are the means through which oxytocin acts as a modulator of neural activity and may ultimately affect various behavioral and psychological processes.

Numerous behavioral studies in rodents have demonstrated oxytocin's potential as an anxiolytic compound. Oxytocin administered subcutaneously to rats prior to an open-field test showed significant reductions in anxiety like behavior (de Wied *et al*, 1987; Klenerova *et al*, 2009; Uvnäs-Moberg *et al*, 1994). Oxytocin given both intracerebroventricularly (ICV) and systemically to mice is anxiolytic in a four-plate test and elevated zero maze test (Ring *et al*, 2006). Oxytocin chronically administered ICV reduced anxious behavior in outbred and high anxiety bred female rats (Slattery and Neumann, 2010; Windle *et al*, 1997). Rats administered oxytocin subcutaneously prior to passive avoidance testing showed reduced latencies for stepping down off the safe platform, which may reflect reduced anxiety (de Oliveira *et al*, 2007; de Wied *et al*, 1987). Post-training subcutaneous oxytocin administration in mice also impaired retention in an inhibitory avoidance task, suggesting oxytocin interferes with memory

consolidation (Boccia *et al*, 1998). Thus, both ICV and systemic administration of oxytocin appear to ameliorate anxiety and aversive learning in a number of paradigms (Rotzinger *et al*, 2010; Viviani and Stoop, 2008).

Recently our lab has used a rodent fear-potentiated startle paradigm to test the effects of systemic oxytocin on fear and anxiety behavior (Missig *et al*, 2010). In addition to analyzing fear-potentiated startle (i.e., the difference in startle between cue-specific fear potentiated startle and startle in the absence of the fear cue), we analyzed the difference in startle before rats were fear conditioned and startle after fear conditioning, called background anxiety (Fig. 1). This work revealed that systemically administered oxytocin reduced the state of background anxiety while leaving cue specific conditioned fear intact. This effect is different from other anti-anxiety compounds which markedly reduce startle during the fear conditioned stimulus (CS) yet leave startle in the absence of the CS unaffected (Davis *et al*, 1993). A series of follow-up experiments revealed that this reduction of background anxiety by oxytocin is likely not due contextual conditioning or a reduction in the ability to startle (Missig *et al*, 2010). This background anxiety may be similar to increased startle responses to unsignaled, unexpected startle stimuli observed in panic and posttraumatic stress disorder patients (Grillon, 2002).

Revealing the central mechanisms of oxytocin's effect on this background anxiety state would be beneficial for both understanding the nature of the background anxiety state itself and for the potential of oxytocin as a treatment for anxiety disorders. In our previous study, oxytocin was given subcutaneously and it was assumed that it enters the brain to directly interact with central oxytocin receptors. However, there is doubt as to whether oxytocin readily crosses the blood-brain barrier in amounts sufficient to exert effects directly in the central nervous system (Ermisch *et al*, 1993; Mens *et al*, 1983). Thus, the focus of the current investigation is to

examine the effect of oxytocin administered ICV 30 minutes prior to fear-potentiated startle testing in order to determine if the effects seen mirror those of systemic administration.

Materials and Methods

Animals:

Male Sprague-Dawley rats weighing between 225-250g were purchased from Charles River Breeders. Rats were pair-housed in shoebox cages in a climate-controlled facility with a 7:00 am – 7:00 pm light/dark cycle and had access to food and water ad libitum. One week after arrival experimental procedures began; all performed between 8:00 am and 4:00 pm. All procedures were in accordance with the US National Institutes of Health *Guide for the Care and Use of Experimental Animals* and approved by the University of Delaware IACUC.

Apparatus:

Eight identical SR Lab ventilated startle chambers with clear Plexiglas cylinders (San Diego Instruments, San Diego, CA) were used for all procedures involving the observation of startle in experiments 1, 2 and 4. One wall of each chamber held an array of three LED lights aligned in parallel that produced 2600 lux and served as the conditioned stimulus (CS). This light intensity does not significantly increase startle in unconditioned rats, but only potentiates startle once it becomes a fear CS (Supplementary Fig. 1). A floor insert made of ten 4-mm diameter stainless steel tubes placed 4 mm apart inside the Plexiglas cylinder was used to deliver foot shocks. Background white noise of 65 dB was continually played throughout all experimental sessions.

For experiment 3, the observation of genital grooming behavior, rats were placed in one of four identical Plexiglas boxes (16.5×12.1×21.6 cm) with metal grid floors (nine stainless steel bars 4 mm in diameter and spaced 1.0 cm apart). This setup was positioned on a Plexiglas stand

inside a fume hood with an overhead fluorescent light which illuminated the entire fume hood. A camera positioned to capture genital grooming behavior in the four chambers was attached to a Dell computer using FreezeFrame software set to the 4 chamber/1 mode (Actimetrics Software).

Experiment 1: Effects of subcutaneously administered oxytocin on background anxiety and fear-potentiated startle.

The startle paradigm used in this and the following studies follows a basic design consisting of three days of startle acclimation, one day of classical fear conditioning, followed by a fear-potentiated startle test session. The first three days of startle acclimation began with a 5-minute acclimation period followed by 30 presentations of startle stimuli ranging from 95dB, 105dB, or 115dB 50ms white noise bursts (10 of each) given in a predetermined pseudo-random order with a 15s inter-trial interval. These sessions served the triple role of acclimating the subjects to the experimental environment, to match subjects into experimental groups, and to permit mean “Pre-Fear” startle scores to be constructed for each subject. These Pre-Fear scores were the mean of the startle amplitudes of all the trials over the three days. Rats with similar Pre-Fear startle amplitudes were assigned to different dose conditions so the various group Pre-Fear startle amplitudes were matched across conditions. Each dosing condition contained 12 subjects, with a total of 48 rats for the experiment. On the fourth day, all rats were classically fear conditioned. A 5-minute acclimation period was followed by 5 pairings of light with a foot shock. Each pairing consisted of 3 second presentation of the light which co-terminated with the 500 ms (0.6 mA) foot shock; the inter-trial intervals ranged from 60 s to 180 s in a pseudo-random order.

Twenty-four hours after fear-conditioning 0, 0.01, 0.1, or 1.0 $\mu\text{g/kg}$ of oxytocin dissolved in saline (Bachem Americas, Torrance, CA, catalog # H-2510) was administered subcutaneously

in the scruff of neck. Animals were weighed on the day of testing and injections were made while the experimenter gently held the animal. Thirty minutes following administration rats were tested for retention of the conditioning in a fear-potentiated startle test. This test consisted of a 5 minute acclimation period followed by 70 startle trials with 15s intervals. The first 10 trials consisted of 95 dB, 105 dB, or 115 dB noise bursts presented in a predetermined pseudo-random order to re-acclimate subjects to the startle stimuli. The next 60 trials consisted of 95 dB, 105 dB, or 115 dB noise bursts presented either in the dark or co-terminating with the 3s light CS presented in a pseudo-random order. For each noise burst intensity 10 trials were presented in the absence of the light and 10 trials were presented in the presence of the light.

Experiment 2: Effects of intracerebroventricularly administered oxytocin on background anxiety and fear-potentiated startle.

Cannula implantation was performed one week following the subjects' arrival to the animal colony. Each rat was anesthetized via an intraperitoneal injection of a ketamine/xylazine cocktail (87mg ketamine and 13mg xylazine/kg) and positioned in a stereotaxic surgical apparatus (Kopf Instruments, Tujunga, California). A guide cannula (22 gauge) obtained from Plastics One (Roanoke, Virginia) measuring 10mm total, with 5mm extending from the base of the threads, was implanted in the left lateral ventricle of each subject (From Bregma: AP: -0.9, LM: +-1.4, DV: -2.4). Following surgery each cannula was fitted with a 28 gauge dummy injector placeholder (Plastics One) that was only removed for infusions, each "dummy" extended 1mm past the end of the guide.

One week following surgery, the behavioral training and testing of fear-potentiated startle procedure began that was identical to Experiment 1 with the exception of drug administration on testing day. Infusions of oxytocin ICV were administered 30 minutes prior to testing on day 5.

Oxytocin was diluted in saline to obtain concentrations of 0.001 μ g, 0.01 μ g, 0.05 μ g, 0.1 μ g, or 1.0 μ g per μ l. Rats were gently held while the dummy was removed and a 28 gauge injector (Plastics One, Roanoke, VA) was inserted into the guide extending 1mm past its tip. The rats were then individually placed in empty shoebox cages to move freely during the 5 minute infusion procedure. All doses were infused at a rate of 1 μ l per minute for 2 minutes, for a total volume of 2 μ l per animal (resulting in final doses of 0.002 μ g, 0.02 μ g, 0.1 μ g, 0.2 μ g, and 2.0 μ g). The rats were then removed from the cages and gently held while the injectors were removed and the dummies reinserted into the guide cannulas. Post-mortem examination of cannula placement was conducted to eliminate subjects with improperly placed guides missing the ventricle. Following this exclusion there was a total of 84 subjects (Saline: N=29; 0.002 μ g OT: N=9; 0.02 μ g OT: N=17; 0.1 μ g OT: N=10; 0.2 μ g OT: N=11; 2.0 μ g OT: N=8).

Experiment 3: Intracerebroventricularly administered oxytocin on genital grooming.

To assure that the 0.002-2.0 μ g ICV doses of oxytocin were behaviorally active, a test of oxytocin-induced genital grooming was conducted following the protocol of Drago et al. (Drago *et al*, 1986). Cannulas were implanted in the same manner as described in Experiment 2. One week following surgery saline, 0.1 or 1.0 μ g oxytocin was infused ICV at a rate of 0.5 μ l/minute for 2 minutes. Thirty minutes later rats were placed into a novel chamber and recorded for 30 minutes. A person blind to the group membership of the rats counted the number of genital grooming bouts. A genital grooming bout was defined as the initiation of genital specific grooming behavior for a minimum of 5 seconds.

Post-mortem examination of cannula placement eliminated misplaced guides so that the subject number totaled 20 (Saline: N=6; 0.1 μ g OT: N=7; 1.0 μ g OT: N=7).

Experiment 4: Effects of Intracerebroventricularly administered high dose of oxytocin on background anxiety and fear-potentiated startle.

Because the oxytocin doses tested in Experiments 2 and 3 did not affect startle but enhanced genital grooming (see Results), we decided to test a higher dose of oxytocin on background anxiety and fear-potentiated startle. The fear-potentiated startle paradigm was identical to that used in Experiment 2, except a high dose of oxytocin (20 μ g) was given. Rats from Experiment 3 and naive subjects were tested. Following post-mortem examination of cannula placements there was a total of 26 subjects: 11 subjects in the saline condition and 15 in the 20 μ g oxytocin condition.

A follow-up study was also conducted to test whether ICV administration of 20 μ g oxytocin suppressed the ability to startle without prior fear conditioning. Cannula implantation surgeries were performed and 7 days following surgery all subjects underwent three startle acclimation sessions as described above. On the fourth day, the rats were not given fear conditioning nor tested for startle. On the fifth day all subjects were given either oxytocin (n=13) or saline (n=10) infusions, then 30 minutes later were tested for acoustic startle in a session identical to the acclimation sessions. One oxytocin-treated rat was removed from the analysis because the injection missed the ventricles.

Data Analysis:

For each component of the startle testing, the amplitudes of the 95, 105, and 115 dB noise bursts were averaged for each rat and used in the statistical analyses. The three startle components are 1) Pre-Fear: the mean startle of each group prior to fear conditioning, 2) Noise: mean startle 24 hours following conditioning in the absence of the light, and 3) Light+Noise:

mean startle 24 hours after fear conditioning in the presence of the CS+ light (Fig. 1). Both Noise and Light+Noise startle responses were taken from a single test session.

The effect of oxytocin in the fear-potentiated startle test was analyzed by a mixed model ANOVA with a between-subject measure of Dose and within-subject measure of fear-potentiated startle (Light+Noise vs. Noise). Post-hoc analysis of a main effect of Dose on startle was performed with a Dunnett's test to compare the various doses of oxytocin to the vehicle (saline). Fear-potentiated startle was analyzed in two different ways – absolute fear-potentiated startle and percent fear-potentiated startle. An absolute fear-potentiated startle score was computed by subtracting the average Noise startle amplitude from the average Light+Noise startle amplitude for each rat. A percent fear-potentiated startle score was computed for each subject by dividing their absolute fear-potentiated startle score by their Noise startle amplitudes. The resulting quotient was then multiplied by 100. Because oxytocin reduced startle during Noise trials, the percent fear-potentiated startle score was used to normalize fear-potentiated startle across groups (Walker and Davis, 2002a). These scores were used to analyze differences in fear-potentiated startle between the oxytocin doses and saline with a Dunnett's test.

Background anxiety was also analyzed by subtracting each subject's Pre-Fear score from its Noise alone score. Since each group was matched to have equal Pre-Fear scores an absolute difference score was sufficient for analysis (Walker *et al*, 2002a). A mixed model ANOVA with a between-subject measure of Dose and within-subject measure of background anxiety (Pre-Fear vs. Noise) was then run on these scores. Post-hoc analysis of background anxiety was performed with a Dunnett's test to compare the various doses of oxytocin to the vehicle (saline).

Genital grooming bouts were defined as the initiation of a stereotypical grooming repertoire specific to the genital region; each bout was defined as lasting a minimum of 5

seconds. General grooming behavior was not included. The number of grooming bouts was analyzed by using a standard two-way ANOVA.

In Experiment 4, startle was also tested in rats that did not receive fear conditioning. The acclimation session (Pre-oxytocin) and Oxytocin startle scores were statistically analyzed in a similar manner as the background anxiety measure of Experiments 1 and 2.

An alpha value of $p < 0.05$ was considered a statistically significant difference for all of the analyses.

Results:

Experiment 1: Systemically administered oxytocin reduces background anxiety following fear conditioning:

Graphs of the data from experiment 1 are shown in figure (2a). Significant main effects were found for Noise vs. Light+Noise trials ($F_{1,44}=127.5$, $p < 0.0001$) and for oxytocin dose ($F_{3,44}=3.78$, $p < 0.017$). However, there was no significant interaction between trial type and dose type ($F_{3,44}=1.44$, ns), indicating no specific effects of oxytocin on fear-potentiated startle. A Dunnett's post-hoc analysis revealed significant differences in startle between saline and 0.1 μg oxytocin ($p < 0.02$) and between saline and 0.01 μg oxytocin ($p < 0.017$) indicating that oxytocin administered systemically at two doses (0.1 $\mu\text{g/kg}$ & 0.01 $\mu\text{g/kg}$) 30 minutes prior to fear-potentiated startle testing significantly reduced overall startle on both Noise and Light+Noise trials. Differences in Percent Fear-Potentiated Startle scores between doses (Figure 2b) were analyzed using a univariate analysis of variance and revealed a lack of an effect of dose on fear-potentiated startle ($F_{3,44}=0.43$, ns).

To evaluate oxytocin's effect on background anxiety a repeated measures analysis of variance was conducted comparing Noise and Pre-Fear trials (Figure 2c). This revealed a

significant main effect of dose ($F_{3,44}=3.06$, $p<0.038$) and a significant dose by trial interaction ($F_{3,44}=2.89$, $p<0.05$). A Dunnett's test background anxiety scores revealed significant differences between saline compared to oxytocin 0.01 μ g/kg ($p<0.036$) and saline compared to oxytocin 0.1 μ g/kg ($p<0.042$). Collectively, these results indicate that systemically administered oxytocin reduces background anxiety, an enhancement of startle to the noise alone that occurs following fear conditioning (Missig *et al*, 2010).

Experiment 2: Intracerebroventricularly administered oxytocin does not have any effect on startle:

To test whether ICV administration of oxytocin also decreases in background anxiety, we examined the effect of 5 doses of oxytocin (0.002, 0.02, 0.1, 0.2, 2 μ g) compared to saline in the same fear-potentiated startle paradigm as Experiment 1. A repeated measures analysis of variance comparing Noise and Light+Noise trials revealed a significant main effect of trial type ($F_{1,78}=93.12$, $p<0.0001$) yet no significant main effect of dose was present ($F_{5,78}=0.63$, ns) nor any interaction ($F_{5,78}=0.57$, ns). Thus, it appears that while fear conditioning was successful and all groups displayed fear-potentiated startle, there was no effect of oxytocin given at any of the 5 doses on Noise alone or Light+Noise startle (Figure 3a). Univariate analysis of variance of percent fear potentiated startle scores confirmed the lack of an effect of oxytocin on fear-potentiated startle at these doses ($F_{5,78}=0.78$, ns) (Figure 3b). Lastly, the measure of background anxiety was examined via a repeated measures analysis of variance comparing the Noise to Pre-Fear scores (Figure 3c) revealing a significant main effect of trial type ($F_{1,78}=61.09$, $p<0.0001$) indicating increased background anxiety, yet there was no effect of dose ($F_{5,78}=0.54$, ns), nor an interaction ($F_{5,78}=0.77$, ns). Thus, it appears that intracerebroventricular infusions of oxytocin at

the doses tested had no effect on diminishing fear-potentiated startle or background anxiety when compared to control animals.

Experiment 3: Intracerebroventricularly administered oxytocin potently enhances genital grooming

The lack an effect of ICV oxytocin on background anxiety at the same doses that were effective when administered subcutaneously is curious because other behaviors have been found to be affected at these ICV doses. To make sure ICV oxytocin has behavioral effects in our hands we examined oxytocin-induced genital grooming. We found oxytocin administered ICV at 2 doses (0.1 μ g and 1.0 μ g) significantly increased genital grooming behavior ($F_{2,13}=32.95$, $p<0.0001$). Furthermore, a Tukey's Honestly Significant Different post hoc analysis revealed saline, 0.1 and 1.0 μ g oxytocin all differed from each other. These results (Figure 4) clearly demonstrate that oxytocin has a profound dose-dependent effect at enhancing genital grooming in a novel environment. The findings indicate that while ICV oxytocin induces genital grooming at certain doses, background anxiety is unaffected at these same doses.

Experiment 4: Intracerebroventricularly administered oxytocin reduces background anxiety at a high dose.

Following experiment 3 two additional groups of animals were tested with infusions of a higher ICV dose of oxytocin (20 μ g) 30 minutes prior to fear-potentiated startle testing. Twelve subjects from experiment 3 were first retested with this higher dose (oxytocin (n=8) and saline (n=6), and upon finding a promising trend 14 new subjects were run (oxytocin (n=7) and saline (n=5). A statistical comparison between the two cohorts did not find any differences in any of the startle measures. Thus, to increase statistical power, the two cohorts were combined for analysis of fear-potentiated startle and background anxiety. A repeated measures analysis of variance

comparing the Noise and the Light+Noise trials (Figure 5a) revealed a main effect of trial ($F_{1,24}=46.57$, $p<0.0001$) and a main effect of dose ($F_{1,24}=9.79$, $p<0.005$). However, there was no interaction present between trial type and dose ($F_{1,24}=0.89$, ns). A univariate analysis of variance of the percentage fear-potentiated startle scores also shown no oxytocin effect ($F_{1,24}=0.72$, ns) (Figure 5b). Lastly, for analysis of background anxiety a repeated measures analysis of variance comparing the Noise and Pre-Fear trials resulted in a main effect of dose ($F_{1,24}=5.96$, $p<0.022$), no effect of trial type ($F_{1,24}=3.35$, $p<0.08$) and also a significant interaction between trial type and dose ($F_{1,24}=5.41$, $p<0.029$) (Figure 5c). Thus, with a high dose of oxytocin (20 μ g), ICV administration appears to reduce background anxiety.

An alternate explanation of the data is also possible. The effect of the high dose of oxytocin delivered ICV might have suppressed the ability to startle or respond to the acoustic stimulus instead of reducing background anxiety. To test whether oxytocin is merely reducing the startle response, rats were not fear conditioned, but tested for startle amplitude with or without oxytocin. Within subject comparisons were made between mean startle responses during acclimation and 30 minutes after saline or 20 μ g oxytocin administered ICV. There were no significant effects of the high ICV dose of oxytocin on startle amplitude (data not shown).

It is also possible that the lack of an effect of oxytocin on startle amplitude was because startle levels were very low in this experiment, and oxytocin may be more effective in reducing high levels of startle like those generated following fear conditioning. We therefore reanalyzed data of Experiment 4 using startle amplitudes induced by the three startle stimulus intensities, 95, 105 and 115 dB noise bursts, individually. The 95 and 105 dB noise bursts had no effect, but at the 115 dB intensity, the 20 μ g ICV oxytocin dose significantly reduced acoustic startle (Fig. 6). There was a significant interaction between acclimation startle (pre-oxytocin) and startle during

20 µg oxytocin ICV vs. saline ICV ($F_{1,20}=6.68$, $p<0.02$). Further post-hoc analysis revealed that startle at 115 dB was significantly decreased by oxytocin ($p<0.009$). The analysis suggests that the 20 µg ICV dose of oxytocin reduced startle responsivity, but not background anxiety specifically, to a loud startle stimulus.

Discussion

The goals of the present studies were to replicate our previous findings showing that systemically administered oxytocin reduces background anxiety without affecting cue-specific conditioned fear-potentiated startle (Missig *et al*, 2010) and examine whether centrally administered oxytocin has similar effects. Background anxiety is thought to be an anxiety state that is not directly related to a cue-specific fear stimulus, but is activated by a conditioned fear stimulus (Missig *et al*, 2010). Subcutaneously administered oxytocin reduced background anxiety at the same doses (0.01 and 0.1 µg/kg) as the original study. Additional experiments in the Missig *et al*. (2010) study demonstrated that the reduction in startle with subcutaneously administered oxytocin was not due to a reduction of contextually conditioned fear nor the animal's ability to startle, but rather appeared to be specific to a reduction in background anxiety. Surprisingly, oxytocin infused ICV in the same range of the systemically administered doses did not reduce background anxiety or any other measure of startle. However, an ICV dose of oxytocin 200-2000 times greater than the subcutaneous doses did reduce acoustic startle without specifically affecting background anxiety or cue-specific fear-potentiated startle. Thus, oxytocin administered into the cerebroventricular system, even at a high dose, did not mimic the reduction in background anxiety of systemically administered oxytocin.

Experiment 1 replicated and extended the findings of Missig *et al*. (2010) by demonstrating subcutaneously administered oxytocin reduced background anxiety when given

and tested 24 hours after fear conditioning. The study found the same oxytocin doses (0.01 and 0.1 $\mu\text{g}/\text{kg}$) reduced background anxiety when given 96 hours after fear conditioning. In both studies the reduction in startle was specific to background anxiety and did not affect cue-specific fear-potentiated startle. Furthermore, the Missig et al. study demonstrated that oxytocin could also be given prior to or shortly following fear conditioning indicating that oxytocin reduces background anxiety not only during testing or expression of fear, but also when fear is being learned or consolidated and then carried over to a later testing session. Oxytocin appears to uniquely and specifically reduce background anxiety, and this amelioration might have long-lasting effects.

In Experiment 2, ICV injections of oxytocin in the same dose range (0.002-2.0 μg) that was effective subcutaneously had no effect on background anxiety nor on any measure of startle. Because this was not expected, we tested whether our ICV injection method was not successfully administering oxytocin into the ventricles. Because grooming is induced by oxytocin and many other neuropeptides (Colbern and Gispen, 1988), in Experiment 3 we therefore tested oxytocin elicitation of genital grooming with 0.1 and 1.0 μg ICV doses. Genital grooming behavior was potently enhanced and replicates previous studies with ICV and peripheral oxytocin administration (Amico *et al*, 2004; Drago *et al*, 1986; Van Erp *et al*, 1993a). This indicated that oxytocin's lack of effect in the dose range tested in the potentiated startle paradigm was not due to our ICV infusion procedures or the integrity of oxytocin administered ICV.

Experiment 4 revealed that oxytocin given at the high ICV dose of 20 μg did reduce background anxiety in a manner comparable to systemic administration. However, testing this dose in non-fear conditioned rats demonstrated that oxytocin reduced acoustic startle elicited with the 115 dB intensity stimulus. This indicates that the 20 μg ICV oxytocin dose reduced

startle without specifically effecting background anxiety, and differs from oxytocin administered systemically in non-conditioned rats which did not reduce acoustic startle elicited by a 115 dB startle stimulus when (Missig *et al*, 2010).

The results beg the question of the locus of action of oxytocin on background anxiety. Comparing ICV and peripheral administration is typically used to differentiate central and peripheral drug effects ((Francis *et al*, 2006; Gibbs *et al*, 1981; Johnson and Epstein, 1975; Simpson, 1975). Conventional wisdom suggests that doses of a psychoactive compound should be less when administered directly into the ventricles than peripheral administration because the compound would distribute throughout the cerebral spinal fluid and diffuse to adjacent and distal brain regions where active sites reside (Francis *et al*, 2006). However, lack of an effect on background anxiety with ICV administration of oxytocin does not support this notion. It is possible that ICV oxytocin needs to be transported back to the periphery to exert its effects on background anxiety. Indeed, ICV drug administration has been compared to a slow intravenous infusion rather than direct administration into the brain (Pardridge, 2005). Cerebrospinal fluid to blood transport is not unique to oxytocin – ICV administration of other peptides and drugs have been shown to be effective peripherally but not centrally (e.g., Aird, 1984; Crawley *et al*, 1991; Francis *et al*, 2006). Concerning mechanisms for transport of oxytocin from cerebrospinal fluid (CSF) to blood, peptide-transport-system-1 moves oxytocin from CSF to blood with a half-time of about 19 min in mice (Durham *et al*, 1991). A transport system for oxytocin across the blood-brain barrier has not been identified to date (Brasnjevic *et al*, 2009), but approximately 0.002% of peripherally administered oxytocin appears to penetrate into brain (Mens *et al*, 1983), which is likely not sufficient to pass through the blood-brain barrier in physiologically significant amounts (Ermisch *et al*, 1985). Given the large difference

in the subcutaneous vs. ICV oxytocin doses (1:200-2000) that suppressed startle (background anxiety subcutaneously vs. acoustic startle intracerebroventricularly), initiation of oxytocin's effects likely begins in the periphery. Speculatively, the effects could be mediated by oxytocin receptors located at the adrenal glands where binding modulates glucocorticoid release (de Oliveria et al., 2007) or at the heart where oxytocin may influence heart rate and blood pressure (Kiss and Mikkelsen, 2005).

Since fear and anxiety are central states, alterations of brain processes are essential for oxytocin to produce its antianxiety effects. Peripheral mechanisms for oxytocin to reduce background anxiety are not known, but could include regulation of autonomic and hypothalamic-pituitary-adrenal axis function (Grippe *et al*, 2009; Kiss *et al*, 2005). Peripheral oxytocin decreases heart rate and blood pressure (Gimpl *et al*, 2001; Petersson *et al*, 1996), alters corticosterone levels (Petersson *et al*, 1999), and may affect blood-brain-barrier permeability by altering the transport of essential substances (Ermisch *et al*, 1993), which then might influence brain processes of emotion and anxiety.

Peripherally administered oxytocin might affect oxytocin brain systems more directly. Peripherally administered oxytocin has been shown to increase neural activity as measured by neuronal Fos expression in oxytocin-producing neurons of the supraoptic and paraventricular hypothalamic nuclei, and areas known to be important for anxiety, such as the central nucleus of the amygdala, locus coeruleus, and parabrachial nucleus (Carson *et al*, 2010) which contain oxytocin receptors (Tribollet *et al*, 1992). Oxytocin synthesizing neurons in the paraventricular hypothalamic and supraoptic nuclei have extensive networks of dendrites and axons which penetrate into the ventricles and subarachnoid space to release oxytocin (Veening *et al*, 2010)). Most of brain areas containing oxytocin receptors are potentially activated by oxytocin released

or injected into the CSF, including the paraventricular hypothalamic nucleus (Gimpl *et al*, 2001; Veening *et al*, 2010). Whereas these oxytocin receptive areas along the ventricles and subarachnoid space may mediate many behaviors, including grooming, following ICV administration of oxytocin (Stivers *et al*, 1988; Van Erp *et al*, 1993b; Veening *et al*, 2010), background anxiety does not appear to be one of these. Nevertheless, site-specific oxytocin injections into the paraventricular hypothalamic nucleus or central nucleus of the amygdala have been shown to decrease anxious behavior (Bale *et al*, 2001; Blume *et al*, 2008). Anxious behavior is also diminished by ICV chronic, but not a single, infusions in outbred and high anxiety bred female rats (Slattery *et al*, 2010; Windle *et al*, 1997). Whether oxytocin delivered ICV chronically is transported to the periphery to be effective or has central effects is unknown.

In conclusion, the reduction in background anxiety by oxytocin appears to be through mechanisms that are either initiated in the periphery or in brain via transport through normal blood vasculature routes. Background anxiety is likely an important dimension of anxiety that is expressed in many mental health disorders. PTSD, panic disorder, and autism spectrum subjects, behaviorally inhibited adolescents, and people going through nicotine withdrawal all display enhanced startle during unpredictable threat, but still have normal cued-fear-potentiated startle (Bernier *et al*, 2005; Brunetti *et al*, 2010; Dichter *et al*, 2010; Grillon and Morgan, 1999; Grillon *et al*, 1998; Grillon *et al*, 1996; Grillon *et al*, 2009; Hogle *et al*, 2010; Morgan *et al*, 1995; Pole *et al*, 2003; Pole *et al*, 2009; Reeb-Sutherland *et al*, 2009; Wilbarger *et al*, 2009). The disorders appear to share a clinical phenotype characterized by anxious apprehension, hypervigilance and exaggerated responsivity during unpredictable, but not predictable, aversive events (Grillon, 2009; Rosen and Schulkin, 1998). While names for this anxious phenotype vary from context fear to contextualization (Grillon, 2002; Liberzon and Sripada, 2008) and may have similarities

to sustained fear (Davis *et al*, 2010; Miles *et al*, 2011) or the persistent increase in ‘baseline’ startle in both Noise and Light+Noise trials coinciding with the first light fear CS presentation (Walker and Davis, 2002b), we think background anxiety – a state not directly related to cue-specific fear, but activated by the cue and sustained beyond the immediate threat – captures the essence of this behavioral phenotype. It has further been conceptualized that fear to specific, predictable threats rides upon this elevated background anxiety in PTSD and panic disorder (Grillon, 2002). Elucidating the mechanisms that oxytocin reduces background anxiety might be important for development of novel therapeutics.

Disclosure/Conflicts of interest

None of the authors has any financial conflicts of interest to report.

Acknowledgments

This work was supported by grant W81XWH-08-1-0182 from the Congressionally Directed Medical Research Programs, U.S. Army Medical Research & Materiel Command.

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Titles and legends to figures

1. Startle responses from three different trial types were used to analyze the effects of oxytocin.

Background anxiety is the increase in startle amplitude in the Noise trials during the fear-potentiated startle test compared with the amplitude during the last acclimation session (Pre-Fear startle). Pre-Fear startle occurs prior to both fear conditioning and oxytocin administration. Cue-Specific fear-potentiated startle is the increase in startle amplitude in the Light+Noise trials compared with the startle amplitude in the Noise trials during the fear-potentiated startle test (Adapted from Missig et al., 2010).

2. The effect of oxytocin administered subcutaneously 30 minutes prior to fear-potentiated startle testing. (a.) Oxytocin at 2 doses (0.01 and 0.1 $\mu\text{g/kg}$) significantly reduced startle during testing. (b.) Percent fear-potentiated startle. There was no effect of oxytocin. (c.) Background anxiety. 0.001 and 0.01 $\mu\text{g/kg}$ doses of oxytocin significantly reduced background anxiety compared to saline. * Indicates statistically significant from saline.
3. Oxytocin administered ICV at 5 doses 30 minutes prior to fear-potentiated startle testing. (a.) Oxytocin ICV had no effect on startle during testing. (b.) Percent fear-potentiated startle was unaffected by oxytocin. (c.) There is no effect on background anxiety at any dose of oxytocin.
4. Oxytocin (0.1 and 1.0 μg) administered ICV 30 minutes prior to the observation of grooming behavior significantly enhanced the number of genital grooming bouts. * Indicates statistically significant from saline.
5. Oxytocin administered ICV at a high dose (20 μg) 30 minutes prior to fear-potentiated startle testing. (a.) 20 μg oxytocin significantly reduced startle. (b.) Percent fear-potentiated startle was unaffected by this high dose of oxytocin. (c.) Background anxiety was significantly reduced by 20 μg oxytocin. * Indicates statistically significant from saline.

6. Effects of oxytocin non-conditioned acoustic startle. (a.) 20 μ g oxytocin administered ICV significantly decreased startle elicited by the 115 dB noise burst (*, $p < 0.009$). Startle elicited by 95 and 105 dB startle stimuli was not affected by oxytocin. (b.) By comparison, oxytocin given subcutaneously at doses effective in reducing background anxiety did not affect acoustic startle in non-conditioned rats. Startle elicited by 115 dB noise burst is shown, but startle elicited by 95 and 105 dB noise bursts was also unaffected. Data from Figure 5 of Missig et al., (2010).

Supplemental Figure 1. Startle in the presence of the 2600 lux light used as a CS did not affect startle in non-conditioned rats. (a.) Startle in non-conditioned rats in the presence or absence of the light was unaffected by the light and was not altered by subcutaneously administered oxytocin. (b.) In contrast, rats that were fear conditioned as described in the Methods displayed fear-potentiated startle when the light CS was present compared to noise alone trials. Also in contrast to non-conditioned rats, subcutaneously administered oxytocin at 0.01 and 0.1 μ g/kg diminished startle. Results shown in (b.) are also presented in Figure 2.

Figure 1

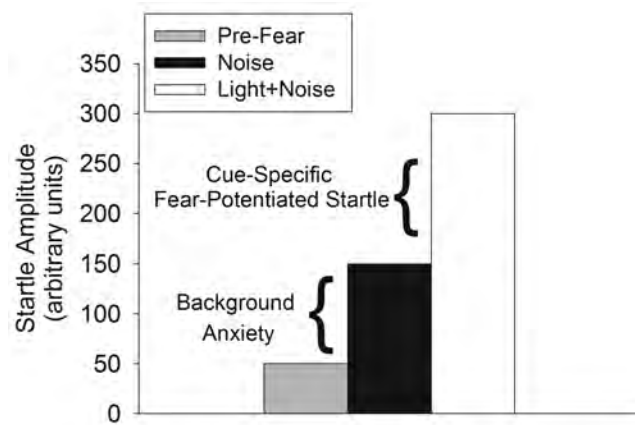


Figure 2

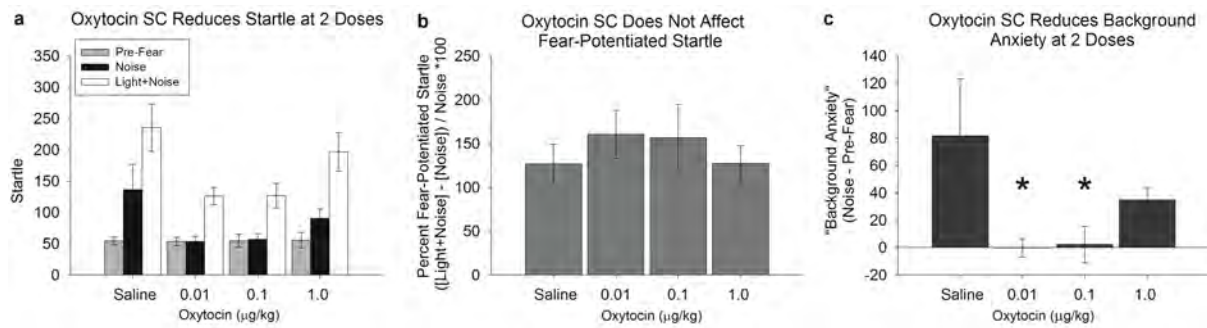


Figure 3

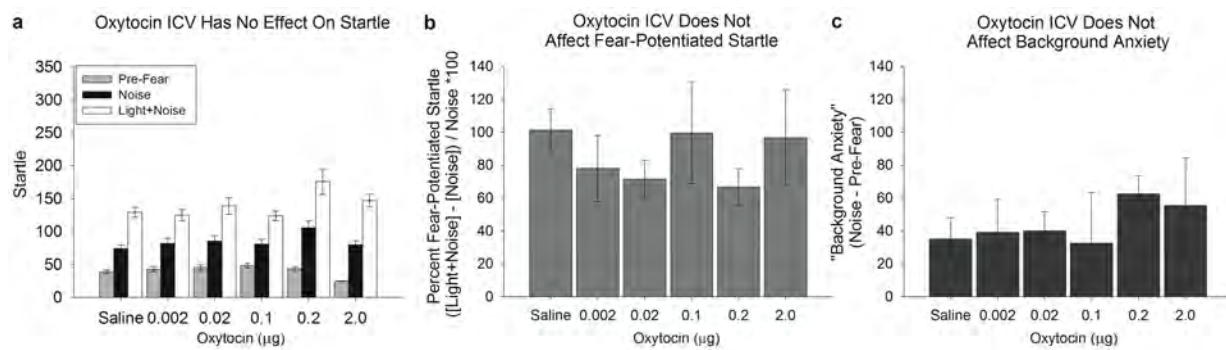


Figure 4

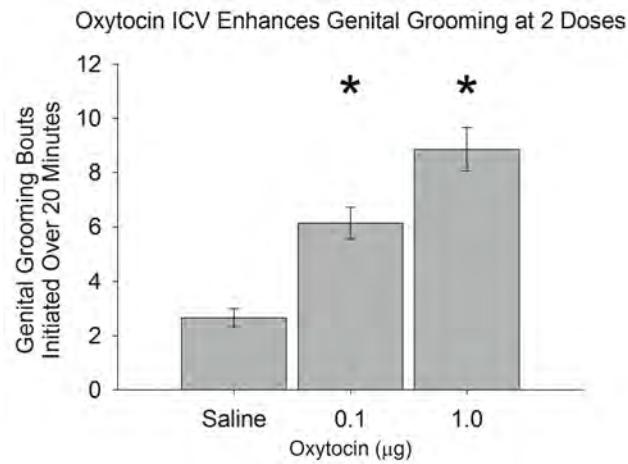


Figure 5

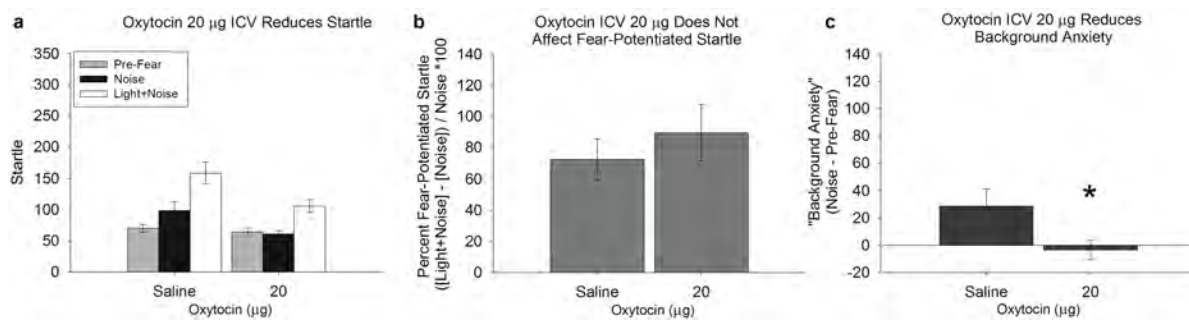
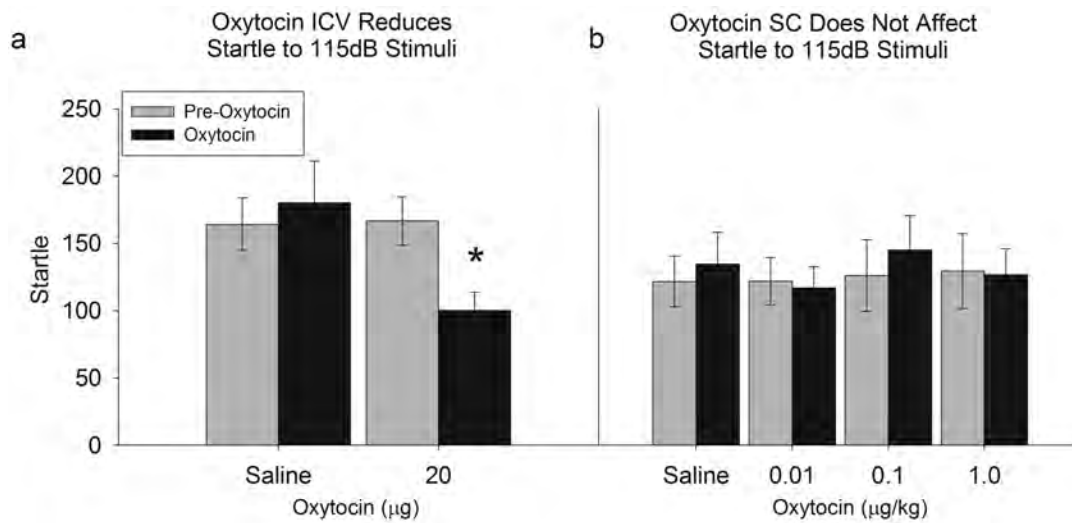
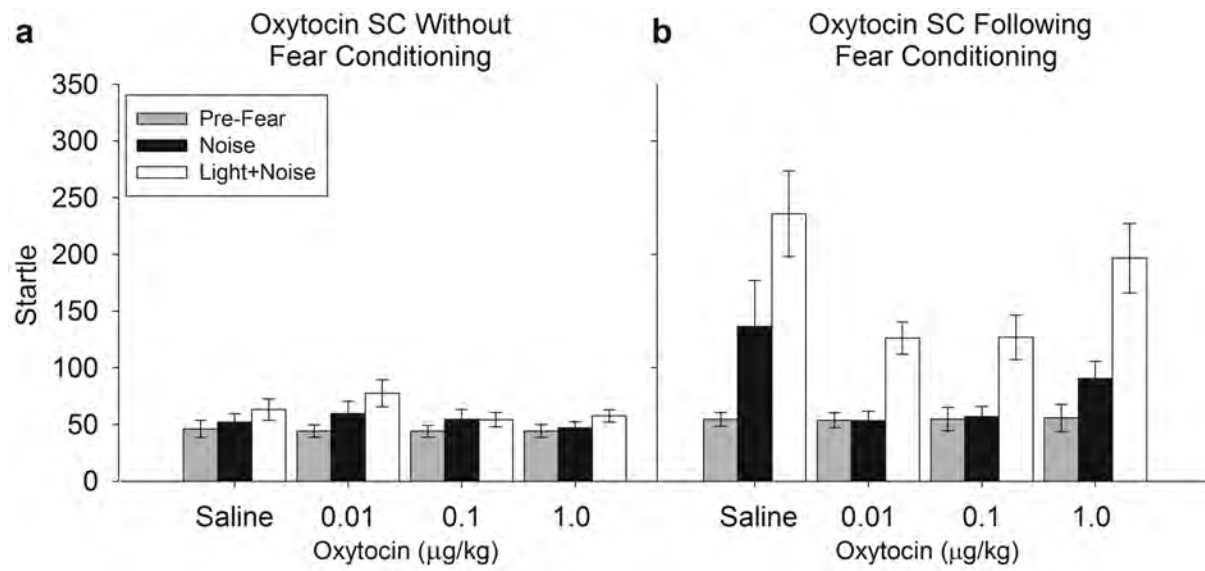


Figure 6



Supplemental Figure 1



Appendix 5:

2010 Society for Neuroscience Abstract #705.24

Systemic, but not intracerebroventricular, administration of oxytocin results in an attenuation of background anxiety in a fear-potentiated startle paradigm

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Oxytocin is a compound long reported to have anxiolytic effects that may depend on an animal's state of anxiety. Recent work in our lab using the fear-potentiated startle paradigm has supported this claim; systemically administered oxytocin (0.01-1.0 μ g oxytocin, s.c.) reduces background anxiety, yet specific conditioned fear is left intact. The effect was not due to oxytocin reducing the rats' ability to startle, nor in reducing contextual fear; rather it appears to relate to generalized anxiety that intermittent CS presentations produce. It remains to be determined if systemically administered oxytocin crosses the blood brain barrier to act in the brain directly, or whether its effects are initiated via interactions in the periphery. To address this question oxytocin was administered directly into the lateral ventricles of rats prior to testing fear potentiated startle. Eighty-eight male Sprague-Dawley rats were implanted with unilateral guide cannula aimed at the left lateral ventricle (ICV). Following recovery, each subject was acclimated to the startle apparatus and acoustic startle stimuli for 3 days. On the 4th day subjects were given standard Pavlovian fear conditioning; 5 pairings of a light and shock. On the 5th day, rats were sorted into four equal groups based on their startle response on the last acclimation day and then tested for fear-potentiated startle under the influence of oxytocin. ICV infusions of oxytocin (ranging from 2ng to 2000ng) were administered 30 min prior to receiving startle stimuli either in the presence or absence of the light. Remarkably, no dose of oxytocin had an effect on any measure of startle. To confirm that oxytocin administered ICV had behavioral effects, genital grooming after ICV administration was tested. Grooming bouts were significantly increased by 100ng and 1000ng oxytocin. Thus, the lack of effects of ICV oxytocin infusion on startle, together with the reduction in startle seen with systemic administration, suggests that oxytocin's effect of reducing background anxiety may be initiated in the periphery. These findings and future preclinical investigations into the mechanisms underlying oxytocin's reduction in background anxiety could lead to novel treatments for anxiety disorders, such as PTSD.

Supported by: Department of Defense, CDMRP, Grant #W81XWH-08-1-0182