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PERIPHERAL OXYTOCIN ADMINISTRATION BUFFERS AUTONOMIC BUT NOT BEHAVIORAL RESPONSES TO ENVIRONMENTAL STRESSORS IN ISOLATED PRAIRIE VOLES

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Abstract

Negative social experiences such as social stressors and isolation influence mental and physical illnesses, including affective disorders and heart disease. Studies focused on socially monogamous prairie voles can provide insight into neurobiological systems that underlie the consequences of negative social interactions. Female prairie voles were exposed to 28 days of social isolation or pairing with a female sibling (control). Animals were administered daily oxytocin (20µg/50µl, sc) or saline vehicle (50µl, sc) for 14 days and exposed to two behavioral stressors (elevated plus maze and resident-intruder test). Brain tissue was collected for analysis of central peptide levels in the hypothalamic paraventricular nucleus. Isolation produced autonomic changes (increased heart rate, decreased heart rate variability) during both acute stressors, and increased anxiety behaviors in the elevated plus maze. Oxytocin prevented the autonomic consequences of the acute stressors in isolated prairie voles, but did not affect the behaviors tested under the present conditions. Oxytocin had no effect on the behavioral or autonomic responsiveness in paired prairie voles. Oxytocin may exert a beneficial effect on autonomic responses to stressors in isolated animals through increasing the numbers of oxytocin-containing neurons and decreasing the numbers of corticotropin-releasing hormone-containing neurons in the paraventricular nucleus. Oxytocinergic mechanisms may serve to compensate for autonomic responses associated with chronic isolation and exposure to both social and non-social acute stressors.

Keywords

Anxiety; Heart rate variability; Hypothalamic-pituitary-adrenal axis; Oxytocin; Paraventricular nucleus; Peripheral administration; Resident-intruder; Social; Stress

INTRODUCTION

Social stressors and the absence of positive social interactions have been associated with several detrimental consequences, including disrupted mood and emotion, increased reactivity of the hypothalamic-pituitary-adrenal (HPA) axis, autonomic imbalance, and central nervous system dysfunction (Anisman and Zacharko 1982; Kim and Kirkpatrick

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1996; Blanchard et al. 2001; McCabe et al. 2002; Ditzen et al. 2007; Grant et al. 2009; Norman et al. 2010). Previous evidence suggests that negative social experiences may mediate the development and maintenance of affective disorders and cardiovascular diseases (Kalinichev et al. 2002; Steptoe et al. 2004; Grippe 2009; Schwerdtfeger and Friedrich-Mai 2009). For instance, in humans feelings of loneliness are associated with behavioral and cardiovascular alterations including increased ratings of hopelessness, reduced self-esteem, and increased diastolic blood pressure (Steptoe et al. 2004). In studies with socially monogamous rodents whose social structure mirrors that of humans (e.g., prairie vole; *Microtus ochrogaster*), long-term social isolation produces several negative behavioral and physiological alterations, including depression- and anxiety-relevant behaviors, autonomic and cardiac dysfunction, and increased reactivity to novel social stressors (Grippe et al. 2007d; Bosch et al. 2009).

The processes that underlie reactions to the social context have been receiving increased attention among researchers (Sgoifo et al. 2001; Cacioppo 2002; McGraw and Young 2009) and the general public (Dingfelder 2006; DeAngelis 2010); however our understanding of the neurobiological mechanisms involved in the consequences of negative (or the lack of positive) social experiences is limited. Evidence from several species, including humans and rodents, has led to the hypothesis that neuropeptides and neurohormones including oxytocin, arginine vasopressin (AVP), and corticotropin-releasing hormone (CRH), play an important role in these associations (Taylor et al. 2006; Bosch et al. 2009; Pierrehumbert et al. 2010). For example, high plasma oxytocin levels in humans have been associated with the absence of positive relationships and lower quality social interactions (Taylor et al. 2006). Mice that are genetically deficient for oxytocin or its receptor have been shown to be hyper-reactive to psychogenic stressors, compared to control groups (Mantella et al. 2004; Takayanagi et al. 2005). In prairie voles, several neuroendocrine disruptions have been observed following short- or long-term social stressors, including increased CRH levels in the hypothalamic paraventricular nucleus (PVN), increased circulating corticosterone levels, increased AVP levels in the hypothalamic supraoptic nucleus, and increased PVN and circulating oxytocin levels (Ruscio et al. 2007; Grippe et al. 2007b).

Studies that focus on the integration of behavioral, cardiovascular, and neural consequences of social stressors using valid animal models will promote a greater understanding of these mechanisms. The prairie vole can be a useful rodent model for investigating mechanisms of sociality and the contribution of the social environment to the regulation of emotion, behavior, and cardiovascular function (2004, 1916). Unlike most rodent species, the prairie vole displays an active engagement in the surrounding social context and forms long-term social bonds similar to humans (Carter et al. 1995; Getz and Carter 1996; Carter and Keverne 2002). Prairie voles also exhibit high levels of parasympathetic (vagal) input to the cardiovascular system at rest, similar to humans and larger mammals, but unlike other laboratory rodents, such as rats and mice (Grippe et al. 2007c).

Given these distinctive characteristics, the prairie vole has been utilized to investigate hypotheses related to the interactions of social behavior, stress, and cardiovascular function. Previous research from our laboratory and others has demonstrated that prairie voles are highly sensitive to social isolation from family members or opposite-sex partners, displaying several depression-relevant behaviors, altered social behaviors, and physiological consequences including altered heart rate (HR) and HR variability, cardiac arrhythmias, sympathovagal imbalance, hyperactivation of the HPA axis, and altered neuropeptide function (Ruscio et al. 2007; Grippe et al. 2007d; Grippe et al. 2008; Bosch et al. 2009; Grippe et al. 2010).

The prairie vole may be a useful model for investigating the role of oxytocin in mediating responses to social isolation. Exogenous oxytocin administration to isolated prairie voles prevented behavioral changes associated with depression as well as several detrimental cardiac consequences including altered HR and HR variability and autonomic imbalance (Grippe et al. 2009). Oxytocin receptors have been identified in cardiac tissue (Jankowski et al. 2004), allowing a direct site of action for this peptide to regulate cardiac function. However, oxytocin also may have actions in the central nervous system that influence both behavioral and physiological responses to stress. For instance, the oxytocin molecule or biologically active fragments of the peptide molecule may gain access to the brain via the circumventricular organs or influence central nervous system functions through indirect mechanisms (see De Wied et al. 1993). To gain a better understanding of the neuropeptide mechanisms that may underlie responses to negative social experiences, the present study examined the influence of oxytocin in the regulation of behavioral, physiological and neural responses to acute stressors in socially isolated prairie voles. Specifically, we investigated the hypotheses that exogenously administered oxytocin would prevent behavioral and autonomic responses to both a social and a non-social stressor in prairie voles previously exposed to social isolation; and that oxytocin also would influence the levels of neuropeptides in the hypothalamic PVN.

MATERIALS AND METHODS

Animals

The subjects consisted of 26 adult (60–90 days of age), female prairie voles, descendants of a wild stock caught near Champaign, IL, with a mean $\pm$ standard error of the mean (SEM) body weight of 44 ± 0.9 g. All animals were maintained on a 14/10 h light/dark cycle (lights on at 0630 h), with a temperature of $25 \pm 1^\circ$ C and relative humidity of 21 ± 4 g/m³. Food (Purina rabbit chow) and water were available *ad libitum*. Offspring were removed from breeding pairs at 21 days of age and housed in same-sex sibling pairs; only one sibling from each pair was used for the experimental procedures described here. All procedures were conducted according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the local University Institutional Animal Care and Use Committees.

Females were chosen for these experiments for several reasons. First, female prairie voles may be especially sensitive to the effects of social stressors (Cushing and Carter 2000; Grippe et al. 2007b). Additionally, female prairie voles do not show a spontaneous puberty or estrous cycle; the ovaries remain inactive until the female has physical contact with a male, allowing for the use of reproductively intact animals without the need for artificially controlling the estrous cycle (Carter et al. 1987). Finally, female rodents are an understudied group both in behavioral and physiological investigations relating to affective regulation and cardiovascular disorders (see discussion in Konkle et al. 2003).

General Experimental Design—Table 1 displays the timeline of procedures employed during the current study. Specific experimental procedures are outlined in the following sections.

Telemetric Transmitter Implantation

Wireless radiofrequency transmitters [Data Sciences International (DSI), St. Paul, MN] were implanted for continuous electrocardiogram (ECG) recordings using procedures similar to those described previously (Sgoifo et al. 1996). Briefly, transmitters were implanted intraperitoneally under aseptic conditions in anesthetized animals during the light period (ketamine 67mg/kg, sc; xylazine 13.33mg/kg, sc; NLS Animal Health, Owings Mills, MD),

and the leads were anchored in place on either side of the heart with permanent sutures. Skin incisions were sutured closed, and subcutaneous fluids were administered as necessary.

All animals were housed for 5 days in custom-designed cages (see Grippe et al. 2007c) which allowed the instrumented animal to interact with its sibling through a divider, but also permitted adequate healing of suture wounds in the instrumented animal. Previous studies (Grippe et al. 2007c; Grippe et al. 2007d) involving surgical implantation of these transmitters in prairie voles have demonstrated that 5 days of recovery in the divided cage is necessary to facilitate adequate healing of suture wounds, but does not significantly affect the animals' ability to re-establish social bonds following this period. Care was taken to allow animals to interact on a limited basis via small holes in the divider. Following 5 days of recovery in the divided cages, animals were then returned to the standard home cages (with the sibling) for an additional 7–10 days before the onset of experimentation. Animals display signs of social bonding (e.g., sitting in side-by-side contact) within the first few hours after being reunited in the standard home cage.

Social Isolation

Following recovery from the surgical procedures, animals were randomly divided into paired (control; $n=13$) or isolated ($n=13$) conditions. Isolated animals were separated from the sibling and housed individually (without sensory cues from the sibling or other conspecific animals) for 4 weeks; paired animals were continually housed with the siblings during this period. Handling, cage changing and measuring of body weight were matched between the groups.

Oxytocin Administration

During weeks 3 and 4 of the isolation/pairing period, animals received one daily subcutaneous injection of either oxytocin (20 $\mu\text{g}/50 \mu\text{l}/\text{vole}$; $n=6$ paired, $n=6$ isolated) or sterile saline vehicle (50 $\mu\text{l}/\text{vole}$; $n=7$ paired, $n=7$ isolated), for a total of 14 injections. Previous evidence demonstrates that multiple peripheral injections of this substance in prairie voles at the dose used here is necessary and sufficient to alter both behavior and autonomic function in a predictable manner. For example, repeated peripheral injections (but not a single injection) of 20 μg oxytocin increases positive social behaviors in adult female prairie voles, including formation of a partner preference (Cushing and Carter 2000). Preliminary studies and published results from our laboratory also indicate that a subcutaneous dose of 20 μg of oxytocin (but not a lower dose) and a dosing schedule of a once-daily injection during the final 2 weeks of a 4-week isolation period (but not a single injection) prevents long-term behavioral and autonomic consequences of social isolation in isolated female prairie voles, including depressive behaviors, increased HR, decreased HR variability, and sympathovagal imbalance (but does not alter the behavior or autonomic function of paired prairie voles) (Grippe et al. 2009). Injections were carried out during the light period between 0900h and 1100h, in a counterbalanced fashion. Immediately following each injection, animals were replaced in the home cages and left undisturbed for approximately 24 hours, until the following scheduled injection time or until the beginning of behavioral tests (described below).

Elevated Plus Maze

Forty-eight hours following the end of the 4-weeks isolation period and the final oxytocin injection, anxiogenic behaviors were measured in the elevated plus maze (EPM) during the light period, using procedures described elsewhere (Pellow et al. 1985). The maze (57 cm height) consisted of two open arms of clear Plexiglas opposite to each other (49.5 $\times$ 10 cm), two closed arms of black Plexiglas with an open roof (49.5 $\times$ 10 $\times$ 30.5 cm), and a center section of clear Plexiglas (10 $\times$ 10 cm). Telemetry receivers were placed under the center and

end of each arm, approximately 15 cm below the maze, to allow for a continuous ECG and activity signal for each animal during the entire test. The animal was placed in the center section and allowed to explore freely for 5 minutes. Animals were returned to the home cage immediately following the test. ECG and generalized activity parameters were recorded continuously prior to, during and following the test. Behaviors were video-recorded during the test, and were subsequently scored by two trained, experimentally blind observers. Animals were coded as entering a section of the maze when all 4 paws crossed into the respective section.

Resident-Intruder Test

Forty-eight hours after the completion of the EPM (96 hours following the end of the 4-week isolation period and the final oxytocin injection), a resident-intruder (RI) test was conducted during the light period, according to procedures described previously (Bosch et al. 2004; Grippe et al. 2007a). The paired or isolated animal (intruder) was placed into the cage of an unrelated and unfamiliar animal of the same sex, age, and approximate size (resident) for 5 minutes. ECG and generalized activity parameters were recorded continuously prior to, during and following the test. Each animal was returned to its home cage immediately following the test. Aggressive behaviors of each resident and intruder [aggressive grooming or posture, swatting, biting, thrusting, pulling, and/or attack behavior (Mitchell et al. 2003)] were video recorded and subsequently scored by two trained, experimentally-blind observers.

Quantification of Radiotelemetric Recordings

Quantification of Telemetric Variables—ECG and activity signals were recorded with a radiotelemetry receiver (DSI; sampling rate 5 kHz for ECG and 256 Hz for activity, 12-bit precision digitizing). These parameters were recorded continuously during all experimental procedures, and were processed and quantified using the vendor software (Dataquest ART Acquisition software version 4.1), according to methods described previously (Grippe et al. 2007c). Briefly, multiple segments of 1–5 minutes of stable, continuous data were used to evaluate HR, HR variability and activity prior to, during and following the behavioral stressors (48–96 hours following the end of the isolation period and the final oxytocin injection). All R-R intervals were manually inspected using custom-designed software [Brain-Body Center, University of Illinois at Chicago, Chicago, IL, (Porges 1985; Porges and Bohrer 1990)], and care was taken to exclude segments of ECG that were confounded by animal movement artifact. HR was evaluated by quantifying the number of beats per unit time (beats per minute, bpm).

The R-R intervals were analyzed for variations to provide two measures of HR variability, including standard deviation of all R-R (normal-to-normal; N-N) intervals [SDNN Index (Task Force of the European Society of Cardiology and North American Society of Pacing and Electrophysiology 1996)] and amplitude of respiratory sinus arrhythmia [RSA (Yongue et al. 1982; Grippe et al. 2007c)]. The SDNN index was evaluated according to procedures described previously (Task Force of the European Society of Cardiology and North American Society of Pacing and Electrophysiology 1996), and is hypothesized to represent the convergence of both sympathetic and parasympathetic innervation to the sino-atrial node.

RSA was assessed with time-frequency procedures (Porges 1985; Porges and Bohrer 1990) that have been validated with humans (Porges 2007), applied to small mammals (Yongue et al. 1982), and modified for the prairie vole (Grippe et al. 2007c). The amplitude of RSA represents the functional vagal impact on the sino-atrial node of myelinated vagal efferent pathways originating in the nucleus ambiguus (see Porges 2007). To deal with the

possibility that violating the assumption of stationarity can distort time series analyses of RSA, the following procedures were implemented: (a) the R–R intervals (heart period) were time-sampled into equal time intervals with a sampling rate of 20 Hz; and (b) the time series were detrended with a moving polynomial filter (Porges and Byrne 1992) that removed variance in the series below 1 Hz for RSA (i.e., 21-point cubic polynomial). The residuals of these procedures were free of aperiodic and slow periodic processes that may violate the assumption of stationarity. A bandpass filter was applied to define RSA by extracting only the variance in the HR spectrum between the frequencies of 1.0 and 4.0 Hz, which has been confirmed through spectral analyses to be the frequency band in which breathing is observed in mammals of similar size to prairie voles (Ishii et al. 1996; Gehrmann et al. 2000; Grippo et al. 2007c).

Behavioral Tests—Cardiac and activity parameters were evaluated using continuous data recorded during the EPM and RI test, using segments of data that were not confounded by movement artifact. Pre-stressor cardiac parameters were derived from ECG data sampled during a period of at least 1 hour of minimal activity [5 counts/minute (cpm) or lower], recorded approximately 2–4 hours prior to the behavioral stressors. Post-stressor parameters were derived from ECG and activity sampled during a period of at least 15 minutes of minimal activity [5 counts per minute (cpm) or lower], recorded approximately 3 hours after each behavioral stressor.

Collection of Tissue and Immunohistochemical Analysis

Forty-eight hours following the final behavioral test (144 hours following the end of the 4-week isolation period and the final oxytocin injection), all animals were anesthetized with a mixture of ketamine (67 mg/kg, sc) and xylazine (13.33 mg/kg, sc) during the light period. Animals were anesthetized within 1 minute of being removed from the housing room, and euthanized via cervical dislocation. Brains were carefully removed and processed with a passive perfusion technique described previously (Cushing et al. 2001; Grippo et al. 2007a). Briefly, brains were immersed in a fixative solution consisting of 4% paraformaldehyde containing 5% acrolein (pH 8.6) for a total of 4 hours. Brains were postfixed for 24 hours in 4% paraformaldehyde, and sunk in 25% sucrose. Tissue was stored in 25% sucrose at 4° C until it was sectioned at 40 μ m on a freezing sliding microtome. Sliced serial brain sections were stored in wells at –20° C, in cryoprotectant antifreeze solution, until analyzed for peptideimmunoreactivity in the PVN.

Serial brain slices (40 μ m) from the PVN were assayed for oxytocin, AVP, and CRH using standard avidin:biotinylated enzyme complex (ABC) immunocytochemistry, according to procedures described previously (Grippo et al. 2007b). Anti-CRH (generously provided by Dr. Ann-Judith Silverman) was used at a concentration of 1:50,000; anti-oxytocin (generously provided by Dr. Mariana Morris) was used at a concentration of 1:300,000; and anti-AVP (MP Biomedicals, Solon, OH) was used at a concentration of 1:200,000. All antibodies were generated in rabbit.

Stained sections were mounted on electrostatically-charged slides, air-dried, dehydrated in a series of ethanol dilutions, cleared with Histoclear (National Diagnostics, Atlanta, GA), and then protected with coverslips using Histomount mounting medium (National Diagnostics, Atlanta, GA).

Images were captured using an Olympus BH-2 microscope (Lombard, IL) equipped with a Pixera Penguin 600CL camera and Pixera Studio 3.0 imaging software (San Jose, CA). The density of oxytocin-, AVP-, and CRH-immunoreactive cell bodies was determined manually in the PVN using a 20 $\times$ objective, according to procedures described previously (Ruscio et al. 2007; Grippo et al. 2007b). Briefly, measurements within the PVN were taken in a

section of the nucleus approximate to Fig. 49 in Paxinos and Watson (2005), where the stained cell bodies take a characteristic shape demonstrated in previous studies (Wang et al. 1996; Ruscio et al. 2007). A standardized sampling area was defined in sections that were matched in rostral-caudal orientation to minimize variability. The total number of immunoreactive cell bodies was counted manually to define a measurement of cell body density within the standardized sampling area. Two to three sections were analyzed from each subject, and results for each animal were averaged across sections. Counts were performed separately for each hemisphere, and the results were averaged between hemispheres. For all subjects, immunoreactive cell body density measures were conducted by two trained, experimentally-blind raters, and the results were averaged between raters. Density measures were therefore averaged across multiple hemispheres, brain slices, and raters to provide an estimate of immunoreactive cell body density for each oxytocin, AVP and CRH in the PVN.

Data Analyses

The data are presented as means $\pm$ (or +) SEM for all analyses, tables and figures. A probability value of $P < 0.05$ was considered to be statistically significant. As described in the following sections: (a) for the behavioral measures, data from animals that did not complete the full behavioral tests were excluded from the analyses; (b) for autonomic measures, periods of ECG involving animal movement artifact were excluded from the analyses; and (c) for central nervous system measures, damaged brain sections were excluded from the analyses.

For repeated measures, data were analyzed with 3-factor mixed-design analyses of variance (ANOVA), with group (paired or isolated) and exogenous treatment (oxytocin or vehicle) as independent variables and the following as repeated dependent variables: (a) HR; (b) SDNN index; and (c) RSA amplitude prior to, during and following the behavioral stressors. Single measures, including (a) behaviors during the stressors; (b) density of PVN oxytocin-immunoreactive cell bodies; (c) density of PVN AVP-immunoreactive cell bodies; and (d) density of PVN CRH-immunoreactive cell bodies, were analyzed using 2-way independent groups ANOVA, with housing group (paired or isolated) and treatment (oxytocin or vehicle) as independent variables. Multiple comparisons were conducted using Fisher's Protected Least Significant Difference post-hoc analyses or Student's t-test with a Bonferroni correction (hypothesis-driven, statistically justified comparisons).

For ease of interpreting the results, the following four group names are used herein: Paired + oxytocin (OT), Paired + vehicle (V), Isolated + OT, and Isolated + V. Results are presented first with a general description of each major finding, followed by a specific description of the statistical test results and associated probability values.

RESULTS

Responses to the Elevated Plus Maze

Autonomic data associated with the EPM were excluded from two animals that did not have complete pre-EPM, EPM, and post-EPM ECG recordings. All data (behavioral and ECG) were excluded from two additional animals that fell off the maze (these tests were immediately aborted). Isolation was associated with anxiogenic behavior during the EPM, with no corresponding change in generalized activity level. However, the anxiogenic behavior was not prevented with oxytocin administration in the isolated group. Isolation also was associated with increases in HR and reductions in SDNN Index (but not RSA) during the EPM; these cardiac alterations were prevented with oxytocin administration in the

isolated group. Oxytocin administration had no significant effects on the behavioral or autonomic responses of paired animals prior to, during or following the EPM.

Behavioral data during the EPM are shown in Table 2. The ANOVA for open arm time yielded a main effect of group [$F(1,20) = 13.48$, $P < 0.002$], but did not yield an interaction. A t-test confirmed that, irrespective of peptide administration, isolated animals spent significantly less time in the open arms of the EPM than paired animals [$t(22) = 3.86$, $P < 0.0004$]. Also, whereas 92% of the paired animals entered the open arm at least once during the 5-minute test, only 50% of the isolated animals entered the open arm ($z = 2.26$, $P < 0.05$).

The ANOVA for closed arm time yielded a housing group x treatment interaction [$F(1,20) = 26.05$, $P < 0.0001$] and main effects of both group [$F(1,20) = 31.72$, $P < 0.0001$] and treatment [$F(1,20) = 4.50$, $P < 0.05$]. A t-test indicated that both isolated groups spent significantly more time in the closed arms of the EPM than both paired groups [$t(22) = 1.91$, $P < 0.03$]; however this behavior was not affected by oxytocin administration ($P > 0.05$). The ANOVA for center section duration yielded a main effect of treatment [$F(1,20) = 4.96$, $P < 0.04$], but did not yield an interaction. Follow-up tests yielded no significant between-group differences in the time spent in the center section ($P > 0.05$ for all comparisons; data not shown).

The ANOVA for general activity level during the EPM yielded no significant effects ($P > 0.05$ for both main effects and interaction).

Autonomic data during the EPM are shown in Figure 1. The ANOVA for HR yielded a main effect of housing group [$F(1,18) = 7.22$, $P < 0.02$] and a main effect of treatment [$F(1,18) = 11.94$, $P < 0.003$]. Post-hoc analyses, with $P < 0.05$ defined as the critical probability value, indicated that the Isolated + V group showed a significantly higher HR than the other 3 groups prior to the EPM ($P < 0.05$ for all comparisons). Additionally, all 4 groups showed a significantly higher HR during the EPM, versus the pre-EPM and post-EPM time points ($P < 0.05$ for all comparisons). The Isolated + V group displayed a significantly higher HR than the other 3 groups following the EPM ($P < 0.05$ for all comparisons).

The ANOVA for SDNN Index yielded a group x treatment interaction [$F(1,18) = 5.65$, $P < 0.03$], a main effect of group [$F(1,18) = 21.27$, $P < 0.0002$], and a main effect of treatment [$F(1,18) = 5.44$, $P < 0.03$]. Post-hoc analyses, with $P < 0.05$ defined as the critical probability value, indicated that the Isolated + V group displayed a significantly lower pre-EPM SDNN Index value than both paired groups and the Isolated + OT group ($P < 0.05$ for all comparisons). The Isolated + V group displayed a significantly lower SDNN Index during the EPM compared to this group's respective pre-EPM value ($P < 0.05$), and the SDNN Index during the EPM in this group was significantly lower than the other 3 groups ($P < 0.05$ for all comparisons). There was no change in SDNN Index during the EPM, compared to pre-EPM or post-EPM time points, in the Paired + V, Paired + OT, or Isolated + OT groups ($P > 0.05$ for all comparisons). Following the EPM, the Isolated + V group's SDNN Index value did not differ significantly from its EPM SDNN Index value ($P > 0.05$), and it was significantly lower than the SDNN Indices of both paired groups at the same time point ($P < 0.05$ for both comparisons).

The ANOVA for RSA amplitude yielded a housing group x treatment interaction [$F(1,18) = 6.88$, $P < 0.02$], a main effect of group [$F(1,18) = 20.39$, $P < 0.0003$], and a main effect of treatment [$F(1,18) = 8.75$, $P < 0.009$]. Post-hoc analyses, with $P < 0.05$ defined as the critical probability value, indicated that the Isolated + V group displayed significantly lower pre-EPM, EPM, and post-EPM RSA amplitudes than the other 3 groups at the same time points ($P < 0.05$ for all comparisons). No significant differences in RSA amplitude were

observed among the Paired + V, Paired + OT, or Isolated + OT groups at any time point ($P > 0.05$ for all comparisons). None of the groups displayed a change in RSA amplitude during the EPM, versus pre-EPM and post-EPM time points ($P > 0.05$ for all comparisons).

Responses to the Resident-Intruder Test

Autonomic data during the RI test were excluded from three animals that did not have complete pre-RI test, RI test and post-RI test ECG recordings. Neither isolation nor oxytocin administration significantly affected behavior during the RI test. However, isolation was associated with altered cardiac responses during and following the RI test, and these responses were partially (but not completely) prevented with oxytocin administration. Oxytocin administration had no significant effects on the behavioral or autonomic responses of paired animals prior to, during or following the RI test.

Behavioral data during the RI test are shown in Table 3. The ANOVA for aggressive behaviors during the RI test yielded no significant effects ($P > 0.05$ for both main effects and interaction). Similarly, the ANOVA for general activity level did not yield any significant effects ($P > 0.05$ for both main effects and interaction).

Autonomic data during the RI test are shown in Figure 2. The ANOVA for HR yielded a housing group $\times$ treatment interaction [$F(1,19) = 8.71$, $P < 0.008$], a main effect of group [$F(1,19) = 33.14$, $P < 0.0001$], and a main effect of treatment [$F(1,19) = 7.70$, $P < 0.01$]. Post-hoc analyses, with $P < 0.05$ defined as the critical probability value, indicated that the Isolated + V group showed a significantly higher HR than the other 3 groups prior to and during the RI test ($P < 0.05$ for all comparisons). The HRs of the other 3 groups were not significantly different prior to or during the RI test ($P > 0.05$ for all comparisons). Additionally, all 4 groups showed a significantly higher HR during the RI test, versus the pre-RI test and post-RI test time points ($P < 0.05$ for all comparisons). Both isolated groups displayed higher HR vs. both paired groups following the RI test ($P < 0.05$ for all comparisons).

The ANOVA for SDNN Index yielded a group $\times$ treatment interaction [$F(1,19) = 7.79$, $P < 0.01$], a main effect of group [$F(1,19) = 11.10$, $P < 0.004$], and a main effect of treatment [$F(1,19) = 4.57$, $P < 0.05$]. Post-hoc analyses, with $P < 0.05$ defined as the critical probability value, indicated that the Isolated + V group showed a significantly lower SDNN Index than the other 3 groups prior to and during the RI test ($P < 0.05$ for all comparisons). The SDNN indices of the other 3 groups were not significantly different prior to or during the RI test ($P > 0.05$ for all comparisons). Following the RI test, both isolated groups displayed significantly lower SDNN indices than both paired groups ($P < 0.05$ for all comparisons).

The ANOVA for RSA amplitude yielded a housing group $\times$ treatment interaction [$F(1,19) = 12.97$, $P < 0.002$], a main effect of group [$F(1,19) = 8.05$, $P < 0.01$], and a main effect of treatment [$F(1,19) = 15.89$, $P < 0.0008$]. Post-hoc analyses, with $P < 0.05$ defined as the critical probability value, indicated that the Isolated + V group showed a significantly lower RSA amplitude than the other 3 groups prior to and during the RI test ($P < 0.05$ for all comparisons), and a significantly lower RSA amplitude versus the Isolated + OT and Paired + V groups following the RI test ($P < 0.05$ for both comparisons). There were no significant differences in RSA among the Paired + V, Paired + OT, or Isolated + OT groups at any time point ($P > 0.05$ for all comparisons).

Paraventricular Nucleus Peptide Responses

Isolation alone did not alter the density of PVN oxytocin- or AVP-immunoreactive cell bodies, but it significantly increased the density of CRH-immunoreactive cell bodies.

Administration of oxytocin led to an increase in density of PVN oxytocin-immunoreactive cell bodies, versus administration of vehicle, in both the paired and isolated groups. Oxytocin administration led to a decrease in the density of CRH-immunoreactive cell bodies in the isolated group, with no detectable difference in CRH in the paired group, for which CRH-immunoreactivity was already low. Administration of oxytocin did not affect AVP-immunoreactivity in the PVN in either paired or isolated groups.

Central nervous system data for statistically significant effects are shown in Figure 3. The ANOVA for density of oxytocin-immunoreactive cell bodies yielded a trend toward a housing group $\times$ treatment interaction [$F(1,22) = 4.28$, $P = 0.05$], and a main effect of treatment [$F(1,22) = 26.97$, $P < 0.05$]. T-tests with a Bonferroni correction, with a probability value of $P < 0.0125$ required, confirmed that both oxytocin-treated groups displayed higher PVN oxytocin levels than both paired groups [Paired + OT vs. Paired + V: $t(11) = 4.31$, $P < 0.0006$; Isolated + OT vs. Isolated + V: $t(11) = 2.88$, $P < 0.007$].

The ANOVA for density of CRH-immunoreactive cell bodies yielded a housing group $\times$ treatment interaction [$F(1,22) = 5.71$, $P < 0.03$], and main effects of group [$F(1,22) = 5.78$, $P < 0.03$] and treatment [$F(1,22) = 4.79$, $P < 0.04$]. T-test with a Bonferroni correction, with a probability value of $P < 0.01$ required, indicated that the Isolated + V group had higher PVN CRH levels than the Isolated + OT [$t(11) = 2.84$, $P < 0.008$], Paired + OT [$t(11) = 2.45$, $P < 0.01$], and Paired + V groups [$t(12) = 3.35$, $P < 0.003$]. No significant differences were found in PVN CRH levels among the Isolated + OT, Paired + OT, or Paired + V groups ($P > 0.05$ for all comparisons).

The ANOVA for density of AVP-immunoreactive cell bodies did not yield any significant main effects or interactions ($P > 0.05$; data not shown).

DISCUSSION

The current study was designed to examine the hypothesis that in female prairie voles oxytocin would modulate behavioral and autonomic responses to acute stressors, and that these effects would vary as a function of the social conditions in which these animals were living. We have demonstrated that chronic isolation in prairie voles serves as a chronic stressor, similar to previous studies (Grippe et al. 2007d; Grippe et al. 2011). These findings also support the hypothesis that exogenous oxytocin can be protective, and is capable of reducing some measures of autonomic reactivity to both social and non-social acute stressors. However, oxytocin treatment did not have a detectable beneficial effect on the behavioral responsiveness to acute stressors in the context of social isolation at the dose and route of administration employed here. Further, the present data indicate that exogenous oxytocin may interact with oxytocin- and CRH-producing cells in the PVN.

The present results suggest that there may be a dissociation between the behavioral and autonomic responses during the EPM in animals that were pre-treated with oxytocin. The autonomic responses of isolated prairie voles differed from paired prairie voles prior to and during this stressor; and also did not recover at the same rate following this stressor. The autonomic changes associated with exposure to the EPM were reduced in isolated animals that received oxytocin, as shown by cardiac rate and rhythm responses comparable to the paired groups prior to, during and following this stressor.

Similar to previous findings (Grippe et al. 2007d), prairie voles exposed to social isolation displayed an anxiogenic response during the EPM relative to paired animals. However, contrary to our initial predictions oxytocin administration did not prevent anxiogenic behaviors during the EPM at the dose and route of administration employed here. This observation differs from some previous human (Heinrichs et al. 2003) and rodent studies

(Uvnäs-Moberg et al. 1994; Windle et al. 1997; Windle et al. 2006; Slattery and Neumann 2010) which have suggested that oxytocin has anxiolytic properties. The experimental protocol employed here differs from these previous studies on several key variables. First, given that oxytocin was administered peripherally in the current study, it is possible that the lack of behavioral anxiolytic effects were due to its inability to access the central nervous system. Second, oxytocin treatments were terminated 48–96 hours prior to behavioral testing, which may have reduced the capacity of the treatment to influence behaviors; although autonomic and neuroanatomical effects of oxytocin were still detected. Third, the effects of oxytocin in the current study may have been partially dependent on the species, reproductive state of the animals, and basal hormone levels. These findings represent behaviors of reproductively-naïve (virgin) female rodents, which differ from some previous studies that have reported anxiolytic effects of oxytocin in men (Heinrichs et al. 2003), male rats (Uvnäs-Moberg et al. 1994), or female ovariectomized rats supplemented with either 17 β -estradiol benzoate (Windle et al. 1997) or with a combination of estradiol and progesterone to mimic the hormonal profile during late pregnancy (Windle et al. 2006). In support of the present results are findings from Neumann et al. (2000), showing an anxiolytic effect of oxytocin in the EPM in pregnant and lactating female rats, but not in virgin rats.

However, in contrast to the present results are those from Slattery and Neumann (2010), showing that chronic intracerebroventricular infusion of oxytocin attenuated anxiogenic behaviors in selectively-bred high anxiety-related behavior Wistar female rats. Given the differences in species, strain, sex, and reproductive status of the subjects (see also Slattery and Neumann 2010), as well as the doses and routes of administration of oxytocin, further investigation of the precise consequences of peripherally vs. centrally administered oxytocin in both humans and animal models will be necessary to determine the mechanism(s) of its beneficial effects on anxiogenic behaviors and autonomic responses to acute environmental stressors.

During the **RI** test, neither isolation nor oxytocin administration altered behavior, including aggressive behavior and generalized activity level. The lack of behavioral changes during this test is consistent with previous studies showing that neither 4 nor 8 weeks of social isolation was associated with altered aggression during this stressor in female prairie voles, despite significant neuroendocrine activation in response to the stressor (versus paired control conditions) (Grippe et al. 2007a; Grippe et al. 2007b). The autonomic profile observed here in isolated animals mirrors the neuroendocrine profile demonstrated previously (Grippe et al. 2007a; Grippe et al. 2007b). At all three time points (prior to, during and following the **RI** test), HR was highest in the Isolated + V group, while SDNN Index and RSA amplitude were lowest in this group. Oxytocin partially prevented these autonomic reactions in isolated animals (refer to Figure 2). Pre-treatment with oxytocin in isolated prairie voles may be associated with a different time course of recovery of autonomic parameters following various stressors, possibly dependent on differential sympathetic and parasympathetic regulation of the heart (see also Norman et al. 2011).

In addition to preventing autonomic responses to the EPM and RI test, oxytocin administration also was associated with changes in the PVN of the hypothalamus. Administration of this peptide was associated with an increase in the density of oxytocin-immunoreactive neurons in the PVN, which may suggest increased synthesis of endogenous oxytocin. Alternatively, this neuroanatomical change could be the result of decreased release of the peptide. Increases in the density of oxytocin-immunoreactive cell bodies were evident in both paired and isolated animals, suggesting that circulating oxytocin may regulate central processes via a feed-forward mechanism, as has been suggested previously (Falke 1989; Yee et al. 2010). Alterations of PVN levels of oxytocin-containing cell bodies may affect a

variety of central target tissues including brainstem regions that regulate the autonomic nervous system, as well as hypothalamic-amygdala targets that may be of particular importance for anxiogenic behaviors.

It is notable that, although oxytocin administration was associated with increased density of oxytocin-immunoreactive cell bodies in the PVN in paired prairie voles, administration of the exogenous peptide did not significantly affect the behavioral or autonomic responses during the two behavioral stressors, nor did it alter CRH levels in the PVN in the paired group. In contrast, administration of exogenous oxytocin was associated with a reduced density of CRH-immunoreactive cell bodies in the PVN in the isolated group. These findings suggest that exogenous oxytocin may have the capacity to block the increase in CRH synthesis that typically accompanies a chronic stressor (e.g., in the absence of supportive social stimuli).

The current results may also be informed from the finding that oxytocin administered intranasally interacts with perceived social support to down-regulate the HPA axis and HR responses in humans (Heinrichs et al. 2003). Additional studies are required to fully understand the release patterns of both oxytocin and CRH from the PVN following social isolation and oxytocin administration. A limitation of the present study is that the central nervous system changes were investigated several days following the final oxytocin injection, with exposure to two behavioral stressors during the intervening period. Although care was taken to ensure that animals had a sufficient amount of time to recover from the disruption of the behavioral stressors prior to the collection of brain tissue, the present findings cannot speak directly to whether some of the changes observed in the PVN are due to complex interactions among social isolation, oxytocin administration, and exposure to acute behavioral stressors.

The precise pathways by which oxytocin may exert a protective effect on autonomic responses to stressors require further investigation. As noted above, oxytocin receptors are present in cardiac tissue (Jankowski et al. 2004), which is a potential site of action for the exogenously administered oxytocin in the present study. However, peripherally-administered oxytocin also may influence central oxytocin function via several mechanisms. The oxytocin molecule or biologically active fragments of the peptide molecule may access the brain through the circumventricular organs (De Wied et al. 1993). Peripheral treatments with this peptide also might act indirectly on the brain via the afferent vagus. Oxytocin receptors have been identified in several central autonomic nuclei, including the dorsal motor nucleus of the vagus, nucleus ambiguus, and nucleus tractus solitarius (see Higa et al. 2002). These are likely sites of action for central oxytocin to influence autonomic regulation of the heart during periods of social isolation and exposure to acute stressors.

The present findings provide evidence that oxytocin may serve to modulate autonomic responses to social isolation, especially within the context of acute stressors. It is possible that peripherally-administered oxytocin influences the central nervous system to also affect the levels or release of oxytocin and CRH within the PVN. Future studies will benefit from an investigation of the specific processes through which this and other neuropeptides influence the integration of behavioral and physiological responses to stressors, including a comparison of peripheral and central actions of these substances. Additional mechanistic studies with translational animal models will improve our understanding of neurobiological and psychological processes that underlie reactions to environmental and social stressors in humans.

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Figure 1.

Mean (+ SEM) HR (Panel A), SDNN index (Panel B), and amplitude of RSA (Panel C) prior to, during, and following the 5-minute elevated plus maze in prairie voles following 4 weeks of social isolation or pairing plus daily exogenous administration of either oxytocin (20 μ g/50 μ l/vole, sc) or sterile saline vehicle (50 μ l/vole, sc), in the following groups: Paired + V (-▲-; n=6), Paired + OT (—▲—; n=5), Isolated + V (-■-; n=6), and Isolated + OT (—■—; n=5). Three-factor mixed-design ANOVA with post-hoc multiple comparisons: * $P < 0.05$ vs. Paired + V, Paired + OT, and Isolated + OT groups at the same time point; † $P < 0.05$ vs. two paired groups at the same time point; # $P < 0.05$ vs. respective EPM value. Abbreviations: ANOVA, analyses of variance, EPM, elevated plus maze; HR, heart rate; OT, oxytocin; RSA, respiratory sinus arrhythmia; SDNN, standard deviation of normal-to-normal intervals; SEM, standard error of the mean, V, vehicle.

Figure 2.

Mean (+ SEM) HR (Panel A), SDNN index (Panel B), and amplitude of RSA (Panel C) prior to, during, and following the 5-minute resident-intruder paradigm in prairie voles following 4 weeks of social isolation or pairing plus daily exogenous administration of either oxytocin (20 μ g/50 μ l/vole, sc) or sterile saline vehicle (50 μ l/vole, sc), in the following groups: Paired + V (- - Δ - -; n=6), Paired + OT (— Δ —; n=5), Isolated + V (- - $\square$ - -; n=7), and Isolated + OT (— $\square$ —; n=5). Three-factor mixed-design ANOVA with post-hoc multiple comparisons: * $P < 0.05$ vs. Paired + V, Paired + OT, and Isolated + OT groups at the same time point; † $P < 0.05$ vs. two paired groups at the same time point; ^ $P < 0.05$ vs. Paired + V and Isolated + OT groups at the same time point; # $P < 0.05$ vs. respective RI test value. Abbreviations: ANOVA, analyses of variance; HR, heart rate; OT, oxytocin; RI test, resident-intruder paradigm; RSA, respiratory sinus arrhythmia; SDNN, standard deviation of normal-to-normal intervals; V, vehicle.

Figure 3.

Brain sections (40 μm) from a representative subject in each group showing the density of immunoreactive cell bodies (number of cell bodies per standardized area; oxytocin, Panel A; CRH, Panel B), and mean (+ SEM) group data (oxytocin, Panel C; CRH, Panel D), in the paraventricular nucleus of the hypothalamus of paired and isolated prairie voles following 4 weeks of social isolation or pairing plus daily exogenous administration of either oxytocin (20 $\mu\text{g}/50 \mu\text{l}/\text{vole}$, sc) or sterile saline vehicle (50 $\mu\text{l}/\text{vole}$, sc), in the following groups: Paired + V (n=7), Paired + OT (n=6), Isolated + V (n=7), and Isolated + OT (n=6). Scale bars = 100 μm . Two-way independent-groups ANOVA with independent-groups, Bonferroni-corrected t-tests for multiple comparisons: * $P < 0.05$ vs. Paired + V, Paired + OT, and Isolated + OT groups; \$ $P < 0.05$ vs. two vehicle-treated groups. Abbreviations: ANOVA, analyses of variance; CRH, corticotropin-releasing hormone; ir, immunoreactive; OT, oxytocin, V, vehicle.