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Oxytocin promotes functional coupling between paraventricular nucleus and both sympathetic and parasympathetic cardioregulatory nuclei

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Abstract

The neuropeptide oxytocin (OXT) facilitates prosocial behavior and selective sociality. In the context of stress, OXT also can down-regulate hypothalamic–pituitary–adrenal (HPA) axis activity, leading to consideration of OXT as a potential treatment for many socioaffective disorders. However, the mechanisms through which administration of exogenous OXT modulates social behavior in stressful environmental contexts are not fully understood. Here, we investigate the hypothesis that autonomic pathways are components of the mechanisms through which OXT aids the recruitment of social resources in stressful contexts that may elicit mobilized behavioral responses. Female prairie voles (*Microtus ochrogaster*) underwent a stressor (walking in shallow water) following pretreatment with intraperitoneal OXT (0.25 mg/kg) or OXT antagonist (OXT-A, 20 mg/kg), and were allowed to recover with or without their sibling cagemate. Administration of OXT resulted in elevated OXT concentrations in plasma, but did not dampen the HPA axis response to a stressor. However, OXT, but not OXT-A, pretreatment prevented the functional coupling, usually seen in the absence of OXT, between paraventricular nucleus (PVN) activity as measured by c-Fos immunoreactivity and HPA output (i.e. corticosterone release). Furthermore, OXT pretreatment resulted in functional coupling between PVN activity and brain regions regulating both sympathetic (i.e. rostral ventrolateral medulla) and parasympathetic (i.e. dorsal vagal complex and nucleus ambiguous) branches of the autonomic nervous system. These findings suggest that OXT increases central neural control of autonomic activity, rather than strictly dampening HPA axis activity, and provides a potential mechanism through which OXT may facilitate adaptive and context-dependent behavioral and physiological responses to stressors.

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Conflict of interest statement

All authors declare that there are no conflicts of interest.

Keywords

Oxytocin; Prairie vole; Stress; Paraventricular nucleus; Brainstem; c-Fos

Introduction

The neuropeptide oxytocin (OXT), along with the closely related arginine vasopressin (AVP), has critical roles in facilitating selective social preferences (Williams et al., 1994; Winslow et al., 1993; Insel et al., 1998; Baribeau and Anagnostou, 2015; Kelly and Goodson, 2014; Lieberwirth and Wang, 2014; Stoesz et al., 2013). In the context of stress, activation of the OXT system may promote social cohesion by encouraging approach toward, affiliation with, and maintenance of proximity between, familiar preferred conspecifics (Carter, 1998; Engelmann et al., 1996; Williams et al., 1992; Witt et al., 1990). Behaviors that bring preferred individuals closer would promote coordinated responses to stressful stimuli, such as sharing vigilance during stressful events that threaten the safety of the social group (Carter, 1998; Carter et al., 2008; Grabowska-Zhang et al., 2012; Griffiths et al., 2004; Seyfarth and Cheney, 2012; Silk et al., 2003; Silk and House, 2011). For example, previous work has demonstrated that OXT regulates social contact and pair bonding in prairie voles (Cho et al., 1999), defensive aggregation in rats (Bowen et al., 2013), and reduces interpersonal distance in humans (Perry et al., 2014).

Previous research has implicated both OXT and social behavior in the regulation of the mammalian autonomic nervous system (Grippo et al., 2007a,b; Grippo et al., 2011; Gutkowska and Jankowski, 2011; Kenkel et al., 2014; Norman et al., 2011). An organism's autonomic state and social functioning dynamically co-regulate each other to adapt to changing environmental conditions (Porges, 2003). Like humans, prairie voles exhibit high levels of sociality and the capacity for selective social bonds (Carter et al., 1995). Prairie voles also have a relatively low resting heart rate, and high levels of parasympathetic activity compared to common laboratory rodents like rats and mice (Grippo et al., 2007a,b).

Here, we examine the hypothesis that exogenous OXT influences the behavioral responses of female prairie voles to a stressor (walking in shallow water) by dampening neural activity in brain regions associated with the HPA axis and autonomic nervous system. Voles were given systemic treatments of OXT or a selective OXT receptor antagonist (L-368,899). We also tested the hypothesis that the behavioral and neural effects of OXT would vary as a function of social housing: voles recovered from the stressor alone or in the presence of a familiar same-sexed social partner (cagemate). Measurements of c-Fos were used as an index of neural activity following the stressor in the paraventricular nucleus of the hypothalamus (PVN), a major site of production of OXT and other stress-related peptides (e.g. AVP, corticotropin releasing hormone) that is critical to the stress buffering and anxiolytic properties of OXT (Smith et al., 2015; Smith and Wang, 2014). The PVN functions in coordination with specific brainstem nuclei known to regulate the autonomic nervous system, including the dorsal vagal complex (DVC), nucleus ambiguous (AMB), and rostral ventrolateral medulla (RVLM) (Herman et al., 1996; Piñol et al., 2012). Plasma corticosterone concentrations provided an index of the functional output of the HPA axis. To

examine possible functional coupling among neural activities in these regions correlations also were run between c-Fos activity in the PVN and in the DVC, AMB and RVLM, and between plasma levels of corticosterone and c-Fos activity in each of these brain areas.

Methods

Animals

Seventy-two pairs of adult, reproductively naïve female prairie voles (*Microtus ochrogaster*) were used in this study. Prairie voles were F3 or F4 descendants of prairie voles originally captured near Champaign, Illinois, and bred in-house. Following weaning at 20 days of age voles were pair-housed with a same-sex littermate, and maintained on a 14 h:10 h light:dark cycle (lights on at 06:00) in a temperature and humidity controlled vivarium. Experiments occurred during the light period. Food (Purina rabbit chow) and water were available ad libitum, and cage changes were performed on a weekly basis. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee.

Experimental paradigm

One prairie vole per pair was randomly designated as the focal vole. Focal voles were momentarily restrained to receive an intraperitoneal (i.p.) injection of oxytocin (OXT), oxytocin antagonist (OXT-A), or sterile isotonic saline vehicle (VEH), and were placed back into their home cage with their same-sex littermate for 30 min. OXT (Bachem, Bubendorf, Switzerland) was dissolved in sterile isotonic saline at a concentration of 100 μg/mL and injected at a dose of 0.25 mg/kg, a dose that has been previously shown to ameliorate autonomic stress responses in voles (Grippo et al., 2009). Oxytocin antagonist (L-368,899, Merck Pharmaceuticals, Kenilworth, NJ) was dissolved in sterile isotonic saline at a concentration of 8 mg/mL and injected at a dose of 20 mg/kg, a dose that has been previously shown to block OXT activity when delivered peripherally (Smith et al., 2010). Treatment with sterile isotonic saline vehicle was delivered at the same injection volume (100 μL). Following injection, focal voles experienced a wet cage stressor (WCS), by being transferred in a plastic cup to a mouse cage containing ~2 cm of water at room temperature. The WCS was designed to model a flooded burrow, mimicking a stressor voles might encounter in their natural habitat. After 5 min of exposure to the WCS, voles were transferred by a plastic cup back into their dry home cage to recover in the presence or absence of their familiar cagemate. After a 60 min recovery period in the home cage voles were euthanized by isoflurane anesthesia (<2 min) followed by decapitation and collection of blood and tissue samples. The reason voles were killed 60 min after termination of the stressor is twofold: 1) plasma corticosterone concentrations are typically elevated by 60 min following an acute stressor, and 2) Fos expression in activated neurons can be detected between 30 and 90 min following neural activation. A schematic depicting the timeline of the experiment is presented in Fig. 1.

Hormone validation of the wet cage stressor

A separate cohort of female voles (n = 12) were used to assess the degree to which a 5 min exposure to a wet cage represents a stressor. Voles were injected with sterile saline, and after 30 min exposed to the WCS. Small volumes (<100 μL) of whole blood were collected to assess the time course of corticosterone release in response to the WCS. Repeated sublingual phlebotomy was performed under light (<1.5%) isoflurane anesthesia immediately following the vehicle injection (Baseline), immediately following the WCS (WCS), 1 h following the WCS (WCS + 1 h), and 24 h following the WCS (WCS + 24 h). Whole blood was collected into heparinized microcentrifuge tubes and kept on ice until centrifugation for plasma collection. Plasma was assayed for corticosterone as reported below (Hormone measurement).

Behavior

Behavior was recorded and scored by two independent observers (>85% concordance) for the first 5 min following reintroduction into the home cage with the familiar cagemate following removal from the wet cage stressor. The frequency and duration of both initiations and receptions of affiliative contact were quantified with Behavior Tracker (v. 1.0). All contact observed during the allotted time period was categorized as affiliative, and never resulted in agonistic interactions. Total time in sedentary social contact was defined as the amount of time that a vole either initiated or received a bout of social affiliation in which both voles were immobile in side-by-side contact. In addition, the frequency and duration of stress-related behaviors were quantified in the first 5 min following removal from the wet cage stressor. An initial survey of behaviors revealed that the non-social behaviors displayed during this early post-stressor period were largely confined to rearing, digging, and autogrooming.

Immunohistochemistry

After behavioral testing and euthanasia, brain tissue was carefully extracted from the skull and placed in a scintillation vial containing a small stir bar and 10 mL of 4% buffered paraformaldehyde (Fisher Scientific, pH 7.5). The vial was placed on a stir plate and allowed to spin for 2 h. Fresh paraformaldehyde was exchanged and the brain was allowed to spin for an additional 2 h. Finally, brains were placed into fresh paraformaldehyde for overnight post-fixation. Brains then were immersed in a 25% sucrose solution and stored at 4 °C until sectioned. Brains were cut in 40 μm coronal plane sections using a freezing sliding microtome (Leica Microsystems, Bannockburn, IL). Sections were stored in cryoprotectant in 24-well plates at −20 °C until processed.

Brains were stained for oxytocin and c-Fos using standard avidin-biotinylated enzyme complex (ABC) immunohistochemistry (Vector Laboratories, Burlingame, CA, USA). Serial sets (every third section) of free-floating tissue sections were rinsed in 0.05 M potassium phosphate-buffered saline (KPBS) for 1 h to remove excess cryoprotectant. Sections next were incubated in 1% sodium borohydride for 20 min at room temperature (RT) to reduce free aldehydes followed by a rinse in KPBS. Sections were then incubated for 15 min in 0.014% phenylhydrazine at RT to block endogenous peroxidase activity and rinsed again in KPBS. Next, sections were incubated in rabbit c-Fos antisera (Santa Cruz Biotechnology

Inc., Santa Cruz, CA, USA) at 1:50,000 dilution in 0.05 M KPBS +0.4% Triton X-100 for 1 h at RT and for an additional 48 h at 4 °C. Sections were rinsed in KPBS before being incubated for 1 h at RT in biotinylated goat, anti-rabbit immunoglobulin G (Vector Laboratories; 1:600 dilution in KPBS +0.4% Triton X-100). Sections were rinsed again in KPBS and then incubated in an avidin–biotin peroxidase complex (45 μL A, 45 μL B per 10 mL KPBS +0.4% Triton X-100; Vectastain ABC kit-elite pk-6100 standard; Vector Laboratories) for 1 h at RT. Sections were rinsed in KPBS and then with Tris-buffered saline. c-Fos immunoreactivity (ir) was visualized by incubation in a solution containing 50 mL of Tris-buffered saline, 1.25 g of nickel sulfate, 41.5 μL of 3% H_2O_2 and 10 mg of nickel-diaminobenzidine (Ni-DAB, Sigma-Aldrich, USA) for 15 min at RT. Sections were rinsed (3 × 5 min) in 0.175 M sodium acetate followed by a series of rinses (3 × 5 min) in KPBS. The dishes containing the tissue were covered with parafilm and stored at 4 °C until the slices were mounted in rostral–caudal order onto superfrost plus slides. The slides were allowed to air dry for at least 24 h. Before coverslipping, the tissue slices, attached on the slides, were dehydrated in ascending ethanol dilutions (70%, 95% and, 100% ethanol respectively), cleared in Histoclear (National Diagnostics, Atlanta, GA, USA), and then coverslipped using Histomount mounting medium (National Diagnostics, Atlanta, GA, USA) with a 24 × 60 mm coverglass (Fisher Scientific, USA).

To determine c-Fos expression in OT staining neurons, another set of sections first underwent the above procedures for c-Fos immunostaining, and then were incubated in rabbit oxytocin antisera at a dilution of 1:300,000 (generously provided by Dr. Mariana Morris, Wright State University, Dayton, OH, USA). The same procedure was carried out as above, except for the absence of nickel sulfate in the chromagen precipitation step, producing an orange-brown stain in contrast to the black c-Fos stain. Double-labeled sections were mounted on gelatin-coated slides and air-dried overnight. Sections then were dehydrated in ascending ethanol dilutions and cleared with Histoclear (National Diagnostics, Charlotte, NC, USA). Slides then were coverslipped with Histomount (National Diagnostics, Atlanta, GA, USA).

Quantification of immunoreactivity

c-Fos immunoreactive (Fos-ir) neurons were measured in a single caudal section of the PVN where staining takes a characteristic heart shape consistent with previous studies (Kenkel et al., 2012). Representative sections containing PVN were selected using the appearance of the hippocampus, third ventricle, and optic tract as landmarks, and these corresponded roughly to coronal Image 64 in the Allen Mouse Brain Atlas (2011). Representative sections containing DMX were selected using the appearance of the central canal in sections caudal to the obex, and corresponded roughly to coronal Image 128 in the Allen Mouse Brain Atlas. Representative sections containing AMB and RVLM were selected by choosing sections caudal and closest to the obex and corresponded roughly to coronal Image 126 in the Allen Mouse Brain Atlas.

For quantification of single-labeled c-Fos ir, slides were coded and images were acquired using a Nikon Eclipse E 800 microscope (Nikon, Tokyo, Japan), a SensiCam camera (Cooke Corp., Auburn Hills, MI, USA) and IP Lab 3.7 computer software (Scanalytics Inc., Fairfax,

VA, USA). All images were acquired using a standardized sampling area in accordance with procedures described previously (Ruscio et al., 2007) to ensure that variability was not a function of variation in defining borders in different subjects. c-Fos ir was quantified with Image J software as the percent of the standardized sampling area within the PVN, DMX, AMB, and RVLM. Standardized parameters were used in each brain region for filtering out nonspecific background immunoreactive staining and highlighting individual stained nuclei.

For quantification of double-labeled OXT-ir/Fos-ir in the PVN-H, images were acquired using a Zeiss AXIO Imager Z1 (Zeiss, Oberkochen, Germany) and counted using the TissueFAXS Acquisition Software with HistoQuest Cell Analysis Module (Tissue Gnostics, Vienna, Austria). Double-labeled neurons were quantified through a cell sorting approach that allows sorting by color and size. Clusters of cytoplasmic orange-brown stain (indicative of OXT staining) within a defined window of size and circularity were identified and outlined in red against a black background. Nuclear black stains (indicative of c-Fos staining) within a defined window of size and circularity were identified and inverted monochromatically to show as white spots. White spots appearing against the black background and within red outlines were counted as double-labeled OXT-ir/Fos-ir neurons.

Hormone measurement

Whole blood samples were collected in <1 min from the trunk following decapitation under isoflurane anesthesia. All blood samples were collected into chilled, heparinized 1.5 mL microcentrifuge tubes and immediately placed on ice. After all samples were collected, they were centrifuged at 4 °C, 822 *g* for 15 min. Plasma was stored at −20 °C prior to measurement of OXT and corticosterone (CORT). OXT and CORT were assayed using commercially available enzyme-linked immunosorbent assay kits (Assay Designs, Ann Arbor, MI; OXT: Cat. # 900-153; CORT: Cat. # 900-097). To give results reliably within the linear portion of the standard curve, plasma was diluted in assay buffer 1:6 for OXT and 1:500 for CORT. The lower limits of detection as per the manufacturer were 15 pg/mL for OXT, and 27 pg/mL for CORT. One sample in the OXT assay (from the OXT pretreatment group) and one sample in the CORT assay (from the SAL pretreatment group) produced > 50% variation between replicates, and were thus excluded due to likely contamination. For the remainder, inter- and intra-assay coefficients of variation were <10% for OXT. The inter- and intra-assay coefficients of variation for CORT were <5% and cross-reactivity with other steroids is <1%.

Data analysis

The plasma CORT timecourse validation of the wet cage stressor was performed with a repeated-measures ANOVA. Effect size was estimated by calculating η^2 using an online effect size calculator (https://www.ai-therapy.com/psychology-statistics/hypothesis-testing/anova?groups=1&parametric=0).

Plasma hormone concentrations, immunohistochemistry, and behavioral data are presented as means ± standard error of the mean by pretreatment group, and were compared by one-way ANOVA with Statview v.5.0.1 (SAS Institute Inc., Cary, NC). If results of the ANOVA were statistically significant ($p < 0.05$), Tukey's range test was conducted to reveal post-hoc

differences between individual pretreatment groups. Effect sizes were estimated with η^2 for ANOVA-based comparisons, and Cohen's d for pairwise t-tests using an online effect size calculator (http://www.campbellcollaboration.org/escalc/html/EffectSizeCalculator-SMD1.php).

Additional analyses were conducted on the basis of promising initial exploration and description of the data. Since activity in the PVN of the hypothalamus (most notably release of corticotropin-releasing hormone) is known to potentiate downstream release of CORT from the adrenal cortex, we examined the correlation between Fos-ir in the PVN and plasma CORT across the pretreatment groups, with the expectation that Fos-ir in the PVN would be positively correlated to plasma CORT concentrations. Correlations were performed by calculating Pearson's r by pretreatment group. Effect sizes were estimated by calculating Fisher's Z_r.

The PVN of the hypothalamus plays an important role in cardioregulatory activity occurring in the brainstem at the dorsal vagal complex (DVC), nucleus ambiguous (AMB), and rostral ventrolateral medulla (RVLM) (Geerling et al., 2010; Piñol et al., 2014; Swanson and Sawchenko, 1980). Since activity in the PVN is known to modulate cardioregulatory brainstem activity, we examined functional coupling of the PVN with each of these brainstem nuclei. In addition, to examine the relative contribution of oxytocinergic neurons in the PVN, we examined the correlation between Fos–OXT double-labeling in the PVN with Fos-ir in the 3 cardioregulatory brainstem regions. All analyses were run with Statview v.5.0.1 (SAS Institute Inc., Cary, NC). Correlations were performed by calculating Pearson's r by pretreatment group. Effect sizes were estimated by calculating Fisher's Z_r.

Results

Corticosterone response to WCS

In comparison to plasma corticosterone concentrations immediately following vehicle injection (i.e. Baseline), voles displayed an elevation in response to the WCS, peaking 1 h after the WCS, and returning back to baseline levels after 24 h (Fig. 7; $F_{3,33} = 9.99$; $p < 0.0001$, $\eta^2 = 0.357$).

Oxytocin

Plasma concentrations of OXT were elevated in voles pretreated with exogenous OXT in comparison to SAL or OXT-A treated voles (Fig. 2: $F_{2,68} = 7.51$; $p = 0.001$, $\eta^2 = 0.181$). Post-hoc pair-wise comparison demonstrated plasma concentrations of OXT in OXT-pretreated voles were elevated approximately 2.5-fold compared to voles pretreated with SAL ($p = 0.001$) or OXT-A ($p = 0.002$).

Corticosterone

Pretreatment with OXT did not dampen the CORT response to the WCS in comparison to groups receiving saline (SAL). Plasma CORT concentrations 95 min following injection (and 60 min following exposure to the wet cage stressor) varied according to pretreatment, with SAL-pretreated voles displaying the lowest average CORT concentration, OXT-A

pretreated voles displaying the highest average concentration, and OXT-pretreated voles displaying concentrations intermediate to these extremes (Fig. 3: $F_{2,68} = 3.24$; $p = 0.04$, $\eta^2 = 0.087$). Post-hoc pair-wise analyses showed that OXT-A pretreated voles had higher plasma CORT concentration compared to SAL pretreated voles ($p = 0.02$).

Fos-ir in the PVN was correlated with plasma CORT concentrations in the SAL-pretreated group ($r_{20} = 0.82$, $p < 0.0001$, $Z_r = 1.16$) and OXT-A-pretreated group ($r_{17} = 0.50$, $p = 0.04$; $Z_r = 0.55$), but not in the OXT-pretreated group ($r_{27} = 0.07$, $p = 0.71$, $Z_r = 0.07$) (see Fig. 3).

Behavior

Voles pretreated with OXT or OXT-A spent a greater amount of time in sedentary social contact (i.e. huddling) than voles pretreated with SAL (SAL: 76.7 ± 13.8 s; OXT: 129.3 ± 14.8 s; OXT-A: 132.0 ± 15.5 s; $F_{2,34} = 4.44$; $p = 0.02$, $\eta^2 = 0.207$). Treated voles did not spend more time initiating social contact (SAL: 22.56 ± 4.0 s; OXT: 43.6 ± 13.2 s; OXT-A: 52.1 ± 19.7 s; $F_{2,34} = 1.35$; $p = 0.27$, $\eta^2 = 0.079$), or receiving social contact (SAL: 54.2 ± 12.4 s; OXT: 85.8 ± 12.9 s; OXT-A: 79.9 ± 19.8 s; $F_{2,34} = 1.46$; $p = 0.25$, $\eta^2 = 0.073$).

Voles that received different pretreatments did not differ in the frequency or duration of non-social stress-related behaviors, which included rearing, digging, and autogrooming, in the first 5 min immediately following removal from the wet cage stressor.

c-Fos immunoreactivity (Fos-ir)

Quantification of Fos-ir was performed to test the hypothesis that OXT pretreatment would dampen stress-induced neural activation in the PVN and cardioregulatory nuclei in the brainstem. Voles pretreated with OXT displayed equivalent levels of Fos-ir compared to SAL controls in the PVN (6.14 ± 0.35 vs 6.18 ± 0.43, $t_{46} = 0.06$, $p = 0.95$, Cohen's d = 0.018), RVLM (1.23 ± 0.23 vs 1.58 ± 0.67, $t_{40} = 0.57$, $p = 0.57$, Cohen's d = 0.18), DVC (1.91 ± 0.18 vs 1.63 ± 0.20, $t_{46} = 1.03$, $p = 0.31$, Cohen's d = 0.30), and NAmb (0.97 ± 0.19 vs 1.48 ± 0.62, $t_{41} = 0.86$, $p = 0.39$, Cohen's d = 0.34).

To index possible functional coupling between the PVN and each of the cardioregulatory brainstem nuclei secondary analyses were performed, examining the correlation in Fos-ir between regions. In general, voles pretreated with OXT displayed statistically significant positive correlations between PVN and cardioregulatory regions while voles pretreated with SAL vehicle and the OXT-A showed a lack of correlation (Fig. 4). The correlation in Fos-ir between the PVN and RVLM displayed a positive relationship in OXT-pretreated voles ($r_{25} = 0.54$, $p = 0.005$, $Z_r = 0.60$), but not in SAL ($r_{13} = 0.065$, $p = 0.83$, $Z_r = 0.065$) or OXT-A-pretreated voles ($r_{14} = 0.02$, $p = 0.95$, $Z_r = 0.02$). The correlation in Fos-ir between the PVN and DVC displayed a positive relationship in OXT-pretreated voles ($r_{26} = 0.41$, $p = 0.03$, $Z_r = 0.44$), but not in SAL ($r_{17} = 0.03$, $p = 0.91$, $Z_r = 0.03$) or OXT-A pretreated voles ($r_{16} = 0.11$, $p = 0.69$, $Z_r = 0.11$). The correlation in Fos-ir between the PVN and NAmb displayed a positive relationship in OXT-pretreated voles ($r_{25} = 0.54$, $p = 0.005$, $Z_r = 0.60$), but not in SAL ($r_{14} = 0.20$, $p = 0.47$, $Z_r = 0.20$) or OXT-A pretreated voles ($r_{14} = 0.03$, $p = 0.92$, $Z_r = 0.03$).

Since the correlation between Fos-ir in the PVN and three structurally and functionally distinct cardioregulatory nuclei in the brainstem was positive and statistically significant only in voles pretreated with OXT, we performed a secondary analysis to examine whether the capacity to recover from the stressor in the presence or absence of their cagemate moderated these positive significant correlations. For all brainstem cardioregulatory nuclei, voles recovering alone displayed statistically significant correlations (Fig. 6; DVC: r_{14} = 0.67, p = 0.008, Z_r = 0.81; NAmb: r_{12} = 0.71, p = 0.009, Z_r = 0.89; RVLM: r_{13} = 0.56, p = 0.05, Z_r = 0.63). Only in the RVLM did voles recovering with their cagemate display a positive statistically significant correlation (r_{12} = 0.57, p = 0.05, Z_r = 0.65). Among voles that recovered with a cagemate, individuals in the top half of the distribution in the total time spent in sedentary social contact displayed positive statistically significant correlations in Fos-ir between the PVN and both NAmb and RVLM (NAmb: r_8 = 0.7, p = 0.05, Z_r = 0.87; RVLM: r_8 = 0.88, p = 0.004, Z_r = 1.38).

An additional secondary analysis was performed to investigate functional coupling between the PVN and adrenocortical output. As shown in Fig. 3, the correlation between Fos-ir in the PVN and plasma CORT concentration displayed a strong positive relationship in SAL pretreated voles (r_{20} = 0.82, p < 0.0001, Z_r = 1.16) and OXT-A pretreated voles (r_{17} = 0.50, p = 0.04, Z_r = 0.55), but not in OXT pretreated voles (r_{27} = 0.07, p = 0.71, Z_r = 0.07).

OXT-ir/Fos-ir immunohistochemistry

After initial analyses of Fos-ir suggested OXT-induced functional coupling between the PVN and cardioregulatory brainstem nuclei, double label immunohistochemistry was performed to determine whether functional coupling of brainstem nuclei involved the OXT-producing neurons in the PVN (Fig. 5). In OXT-pretreated voles, the number of neurons double-labeled for OXT and Fos in the PVN was not significantly correlated with the number of c-Fos-positive neurons in the DMX (r_{28} = 0.26, p = 0.18, Z_r = 0.27), NAmb (r_{24} = 0.18, p = 0.38, Z_r = 0.18) or RVLM (r_{25} = 0.11, p = 0.60, Z_r = 0.11). Overall, the number of double-labeled neurons in the PVN did not differ between pretreatment conditions ($F_{2.65}$ = 2.51; p = 0.09) or recovery conditions ($F_{1.65}$ = 1.20; p = 0.28), and these factors failed to interact to produce significant differences ($F_{2.65}$ = 1.25; p = 0.29).

Discussion

The present experiment examined the effect of an acute, peripheral OXT pretreatment on neural, hormonal, and behavioral responses to a stressor, in both social and non-social contexts. OXT treatments have been suggested as therapies for a wide variety of neuropsychiatric disorders; especially those associated with exaggerated reactivity in response to a challenge (Bakermans-Kranenburg and van IJzendoorn, 2013; Harris and Carter, 2013; Macdonald and Feifel, 2013). However, the mechanisms through which OXT affects behavior or emotional reactivity remain incompletely understood.

In this study, voles that were injected with OXT and recovered from a stressor in the presence of their partner engaged in lower levels of escape-related anxiety behavior. However, OXT pretreatment failed to dampen the HPA response to the stressor; rather, statistical trends demonstrated that both pretreatment with OXT and the presence of a

familiar partner during recovery were associated with heightened CORT concentrations. Furthermore, the typically stress-responsive hypothalamic PVN (Ceccatelli et al., 1989; Cullinan et al., 1995; Pacak and Palkovits, 2001) did not exhibit a decrease in Fos-ir in response to OXT pretreatment as would be expected if OXT were to strictly dampen stress-induced HPA axis activity (Windle et al., 2004). Instead, OXT pretreatment led to a pattern of functional coupling between the PVN and cardioregulatory brainstem nuclei, and a statistical uncoupling between the PVN and plasma CORT concentrations. Blocking the OXT receptor with an OXT antagonist (OXT-A) reversed each of these relationships. Cells double-labeled for OXT and Fos did not correlate with Fos-ir in the brainstem regions examined, suggesting that PVN neurons that functionally couple to brainstem cardioregulatory neurons under OXT administration are not solely OXT-producing neurons. Taken together, these findings point toward a more nuanced role for OXT in the regulation of stress responses, one in which OXT enhances adaptation by functionally coupling various types of cells in the hypothalamic PVN, a brain region that influences both sympathetic and parasympathetic autonomic function, to other brainstem regions, including those that regulate the autonomic nervous system.

The response to environmental threats and challenges often occurs in a social context, and OXT has been proposed to play an important role in facilitating adaptive social behavior in the face of stress (Gordon et al., 2008; Smith and Wang, 2014; Tops et al., 2014). Consistent with past work in voles (Witt et al., 1990) and other species (Bowen et al., 2013; Kojima and Alberts, 2011), OXT facilitated affiliative social behavior, with OXT-pretreated voles spending a greater amount of time in sedentary social contact with their sibling cagemate following stressor exposure. Notably, while OXT pretreatment increased the total time spent huddling, it did not impact the number of times injected voles initiated or received affiliative contacts, suggesting that in the face of this challenge additional OXT did not influence the motivation to seek out social partners, nor the attractiveness of the treated vole. This tendency for trusted individuals to group together in dangerous situations, which has been termed “defensive aggregation” (Bowen and McGregor, 2014), allows individuals to take advantage of their social context for the purposes of enhancing survival when it is threatened. Together these data are consistent with the notion that OXT promotes a specific form of prosociality in the context of stress, one in which safe, familiar individuals huddle together to share the burden of hyper-vigilance provoked by dangerous circumstances.

Functional coupling between PVN and brainstem areas under OXT pretreatment was most evident in voles recovering alone, supporting the hypothesis that in the absence of social partners, OXT may serve as a physiological surrogate for social support (Carter, 2014). However, among voles that recovered with familiar partners, functional coupling also was evident in those that spent a greater amount of time in close social contact, suggesting that familiar social partners only provide adaptive benefits during stressful situations if they display a mutual ability to provide each other with a sense of safety as evidenced through greater time spent in sedentary side-by-side contact (see Fig. 6). These findings reinforce the notion that the mere presence of a familiar individual is not sufficient to induce enhanced neural connectivity, and that the quality of the social relationship influences functional coupling between areas regulating stress responses and autonomic activity.

Pretreatment with OXT resulted in heightened plasma OXT concentrations 95 min following the injection. Further research and improved methods are needed to distinguish whether measured increases in OXT were of endogenous origins, or residuals of the exogenous treatment. However, given the reportedly short, circulating half-life of OXT, this might have reflected feed-forward effect of exogenous OXT on the release of endogenous peptides, as has previously been suggested (Falke, 1989; Moos et al., 1984). In voles chronic administration of OXT has been shown to increase OXT synthesis in the hypothalamus (Grippo et al., 2011).

The wet cage stressor was designed to provide the voles with a well-defined challenge. But like many types of acute stressors, an adaptive response to this experience also involved physical mobilization and the requisite mobilization of energetic resources. The trend toward an increase in CORT associated with either OXT treatment or recovery from a stressor in the presence of a partner was not robust. However, the lack of an OXT- or partner-induced reduction in CORT, together with our anatomical data, is not in line with the widely discussed hypothesis, drawn from research in rats (Petersson et al., 1999; Windle et al., 2004, 1997; Babygirija et al., 2012; Blume et al., 2008; Bülbül et al., 2011; Jurek et al., 2015; Zheng et al., 2010), mice made mutant for OXT (Amico et al., 2008), prairie voles (Smith et al., 2015; Smith and Wang, 2014), and humans (Heinrichs et al., 2003; Jong et al., 2015), that OXT and social support exert protective effects primarily by buffering against activation of the HPA axis. Instead, our data suggest that when presented with a stressor that calls for physical and energetic mobilization, administration of exogenous OXT does not prevent what could be an adaptive rise in glucocorticoid levels. Previous work in rats has demonstrated OXT-induced potentiation of the HPA axis (Gibbs, 1985; Gibbs et al., 1984) in contexts such as forced swim, that necessitate a mobilized behavioral response. Thus, taken together these findings suggest that the impact of OXT on HPA axis activity is flexible according to the nature of the stressor, and may facilitate survival by encouraging the maintenance of social proximity even when animals are energetically prepared for mobilization.

Evidence of a functional uncoupling of PVN Fos-ir from CORT levels under OXT pretreatment suggests the more specific hypothesis that exogenous OXT administration might release an inhibitory control exerted by the neurons in the PVN over activity of the HPA axis, especially in the face of stressors that require mobilization. However, further research on the neuroendocrine basis of social behavior during stress is needed. Indeed, prior work demonstrating that stressors and CORT facilitate the formation of selective social preferences (DeVries et al., 1996, 1995) points to the need to untangle the complex interactions between OXT and CORT in these contexts.

Limitations and future directions

The present study is an initial step toward describing the possible role of OXT in the autonomic and behavioral management of stressful experiences. However, the outcome of this study could be specific to the method, timing and doses of OXT or OXT-A used here, and may not generalize to other species. Both OXT and OXT-A could influence other systems, including those that rely on AVP, CRF and so forth.

Administration of OXT-A was capable of blocking functional connectivity between the hypothalamic paraventricular nucleus and cardioregulatory brainstem nuclei, but it did not result in the expected dampening of sedentary social contact. This points to an important limitation of this study, and many studies of the biological basis of social behavior in animals, namely, the lack of fine-grained dissections of social behavior. While sedentary social behavior is an important behavioral outcome that helps contextualize the manner in which the stressed animal copes with the stressor, it provides limited insights. Alternatively, sedentary social behavior may be determined by different, even opposing, socioaffective states. Analyses of social behavior utilizing new technologies to automatically track multiple animals through a variety of contexts may be able to produce richer descriptions of social behavior that capture the subtleties characteristic of social relationships (Hong et al., 2015; Ohayon et al., 2013).

The present mode of treatment used peripheral injections and does not permit specification of the site of action of the peptide. The method is comparatively non-invasive, but also may not allow maximal exposure of some brain areas to the peptide, since the blood–brain barrier may prevent or slow the passage of exogenous OXT into the nervous system. However, brainstem nuclei are less protected by this barrier (Maolood and Meister, 2009). In addition, OXT binds to peripheral receptors (OXT or AVP) and presumably transmits a signal that travels to the CNS through afferent nerve fibers. Thus, even if the injected molecules do not reach the CNS, as with peripheral cytokines, OXT may induce behavioral and anatomical effects, such as those seen here, via the afferent vagus (Goehler et al., 1999). Peripherally administered OXT also may reach the CNS via circumventricular organs such as the area postrema and subfornical organ, known to play a role in neuropeptidergic signaling as well as regulation of ANS activity (Ferguson et al., 1990; Ferguson and Katkin, 1996; Ferris et al., 2015; Komisaruk and Sansone, 2003; Rudinger et al., 1969; Vivas et al., 2014).

Future work is needed to characterize whether PVN neurons functionally coupled to brainstem autonomic nuclei involve other systems, such as AVP or CRF, whose neurons are abundant in the PVN. Based on our correlational approach, functional coupling between the PVN and brainstem cardioregulatory regions was apparently not driven by activated cells synthesizing OXT, thus suggesting the involvement of other systems. For example, these findings may be consistent with recent work demonstrating the involvement of glutamate receptors in mediating downstream effects of OXT-receptor activation (Piñol et al., 2012).

The present studies focused on brainstem regions that are part of the autonomic nervous system. Our findings implicate OXT in the regulation of both the sympatho-adrenal axis and the parasympathetic nervous systems and suggest that cardioregulatory centers in the brainstem can respond to peripherally administered OXT. Furthermore, they suggest that the directional effects of OXT are context-dependent and adaptive, promoting autonomic, behavioral and endocrine regulation under circumstances in which mobilization promotes survival. Because of increasing interest in the clinical usage of OXT (Harris and Carter, 2013) the present findings support the need for more research on the mechanisms through which OXT can affect behavioral and physiological responses to environmental challenges.

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Fig. 1.

Schematic depicting the experimental timeline. Oxytocin (OXT), oxytocin antagonist (OXT-A), or sterile isotonic saline (SAL) injections were administered intraperitoneally (i.p.) to one vole in a same-sex sibling pair. After 30 min in the home cage with their cagemate, injected voles were placed in a wet cage stressor for 5 min, and then returned to their home cages where they either remained alone or with a familiar cagemate. All voles were euthanized and tissue samples collected 60 min following cessation of the stressor (95 min following the injection).

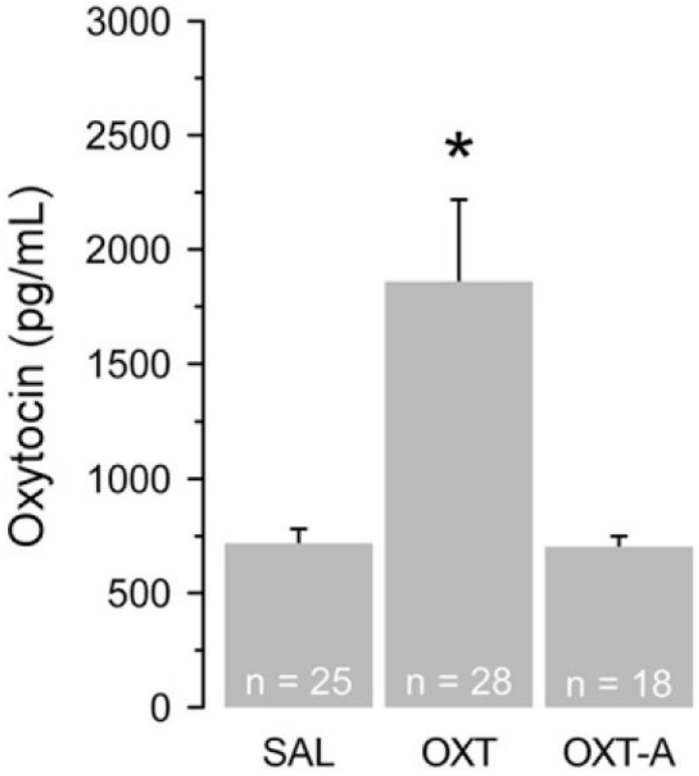

Fig. 2.

Mean (±SEM) plasma oxytocin concentrations measured by EIA. Female prairie voles received an intraperitoneal (i.p.) injection of sterile saline (SAL), oxytocin (OXT), or an oxytocin antagonist (OXT-A), and recovered from the wet cage stressor in their home cages either alone or with their partner. Housing conditions did not affect outcome and are combined here. *$p < 0.005$ vs Saline and OXT-A.

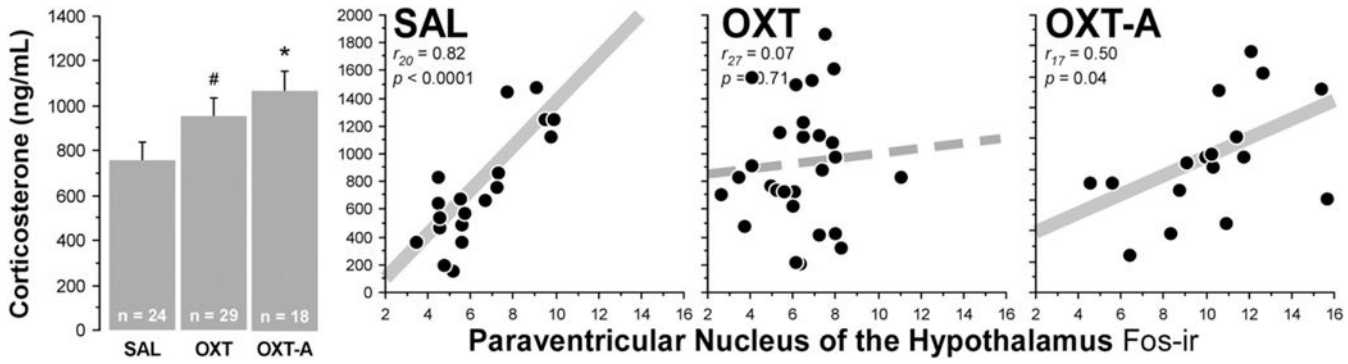

Fig. 3.

A. Mean (±SEM) plasma corticosterone concentrations measured by EIA following injection (i.p.) of sterile saline (SAL), oxytocin (OXT) or oxytocin antagonist (OXT-A). *p < 0.05 vs Saline. $^{\#}p$ < 0.10 vs Saline. B. The positive correlation between Fos-ir in the paraventricular nucleus of the hypothalamus (PVN) and plasma corticosterone seen under control conditions (SAL) was absent in animals receiving OXT, but present in animals receiving an antagonist (OXT-A). Thick gray lines indicate significant correlations (p < 0.05). c-Fos immunoreactivity (Fos-ir) in the PVN was not available for all animals for which blood was measured.

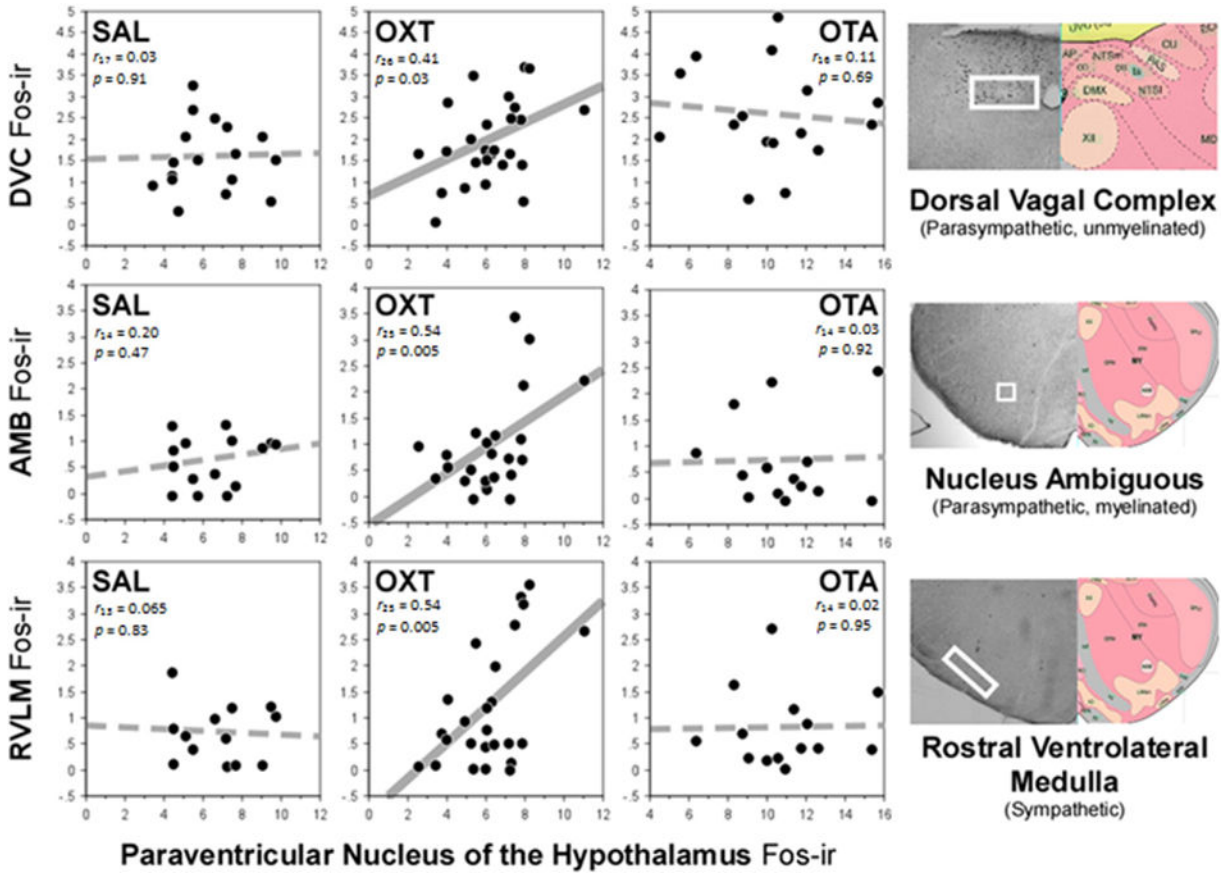

Fig. 4.

Neural activation (indexed by Fos-ir) in the PVN (abscissa) is significantly correlated with activation in brainstem cardioregulatory areas (ordinate) in animals receiving OXT, but not those receiving SAL or OXT-A. Fos-ir in the dorsal vagal complex (DVC; ordinate, top row), nucleus ambiguous (AMB; ordinate, middle row), and rostral ventrolateral medulla (RVLM; ordinate, bottom row) was used to index neural activation in cardioregulatory brainstem nuclei. Saline controls (SAL) are represented in the first column, oxytocin-treated voles (OXT) are represented in the second column, and oxytocin antagonist-treated voles (OXT-A) are represented in the third column. On the right, representative photomicrographs of c-Fos immunoreactivity with atlas overlay (Allen Mouse Brain Atlas); white boxes represent the quantified region. Thick gray lines indicate significant correlations ($p < 0.05$).

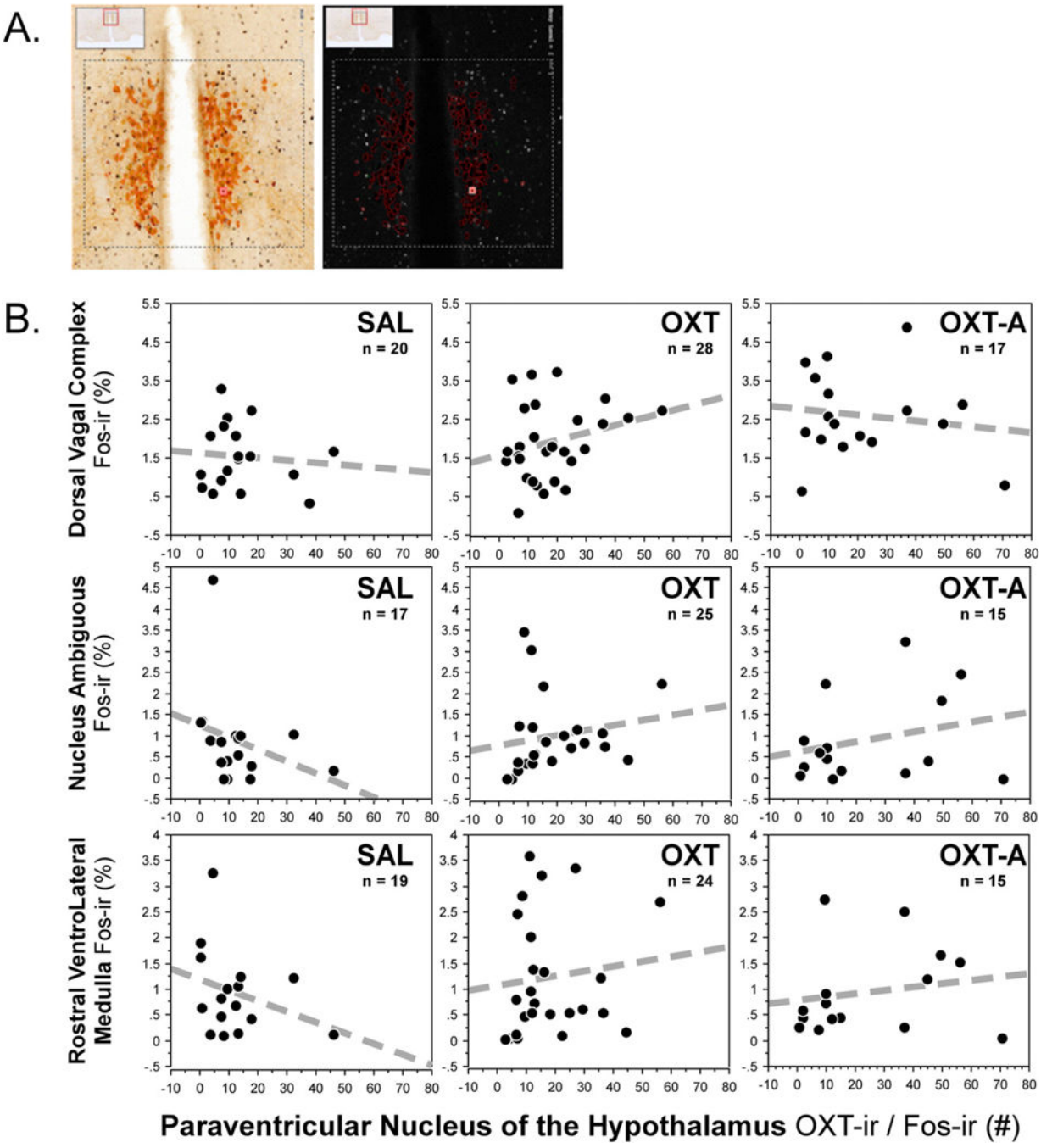

Fig. 5.

Functional coupling of PVN and brainstem cardioregulatory regions by OXT administration (as shown in Fig. 4) may not involve OXT-secreting neurons in the PVN. (*Top*) Representative photomicrograph of OXT-ir/Fos-ir double-immunolabeling as analyzed with the TissueFAXS/HistoQuest cell sorting system which accurately sorts cells by chromogen. (*Bottom*) Correlation matrix depicting associations between percentage of OXT-ir/Fos-ir double-immunolabeled cells in the PVN (abscissa, all graphs), and Fos-ir in the dorsal vagal complex (DVC; ordinate, top row), nucleus ambiguous (AMB; ordinate, middle row), and

rostral ventrolateral medulla (RVLM; ordinate, bottom row). Saline controls (SAL) are represented in the first column, OXT-treated voles are represented in the second column, and OXT-A-treated voles are represented in the third column.

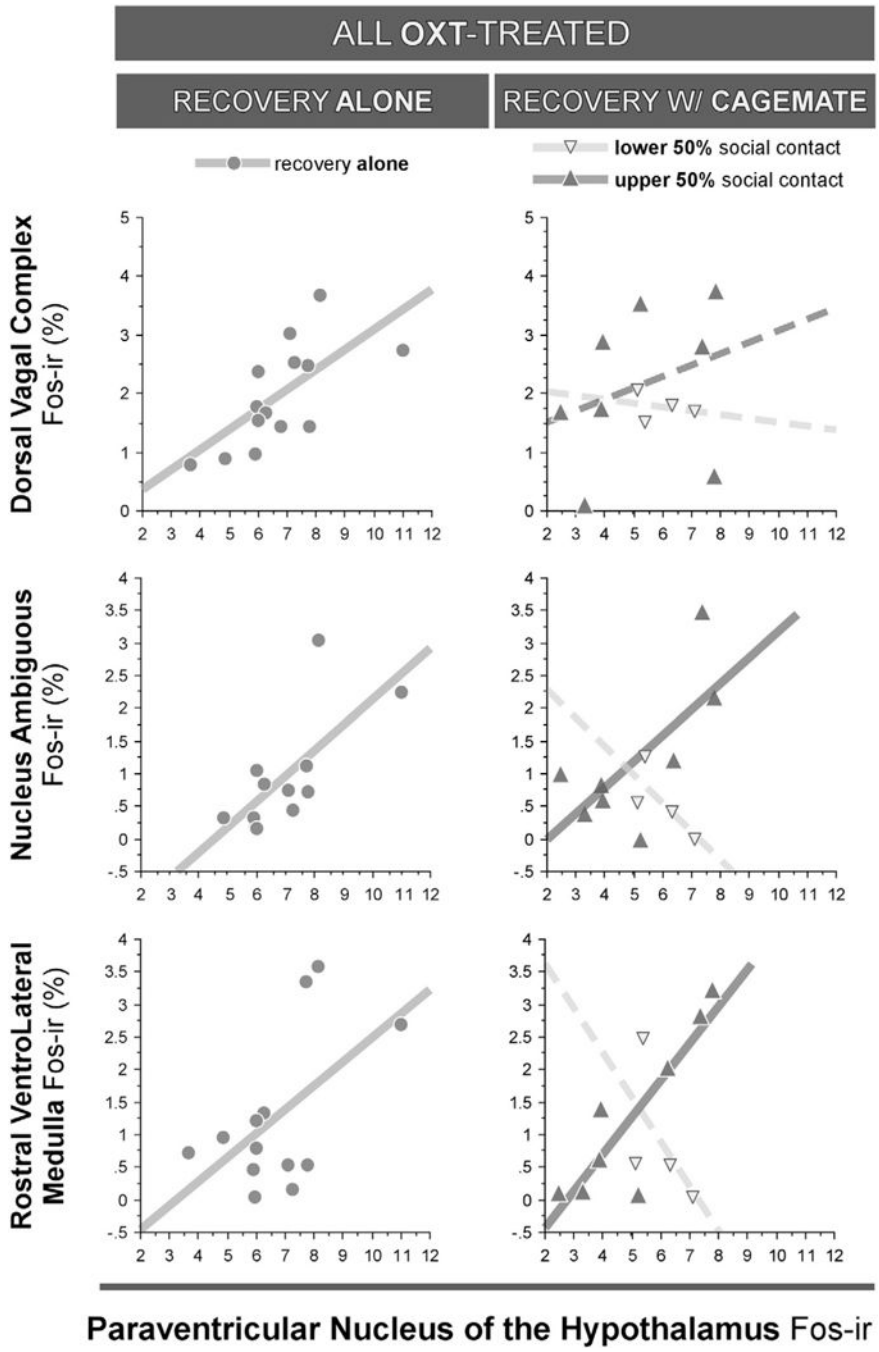

Fig. 6.

Functional coupling of PVN and brainstem cardioregulatory regions by OXT administration is strongest in voles that recovered alone (*left column*). For voles that recovered with a partner (*right column*), functional coupling of PVN and brainstem cardioregulatory regions by OXT administration is only seen in voles in the upper 50% of social contact. Voles were split along the median into the upper 50% (dark gray symbols) and lower 50% (light gray symbols) based upon the time spent in social contact in the 5 min following the wet cage

stressor. Solid and hashed lines indicate significant ($p < 0.05$) and non-significant ($p > 0.05$) correlations, respectively.

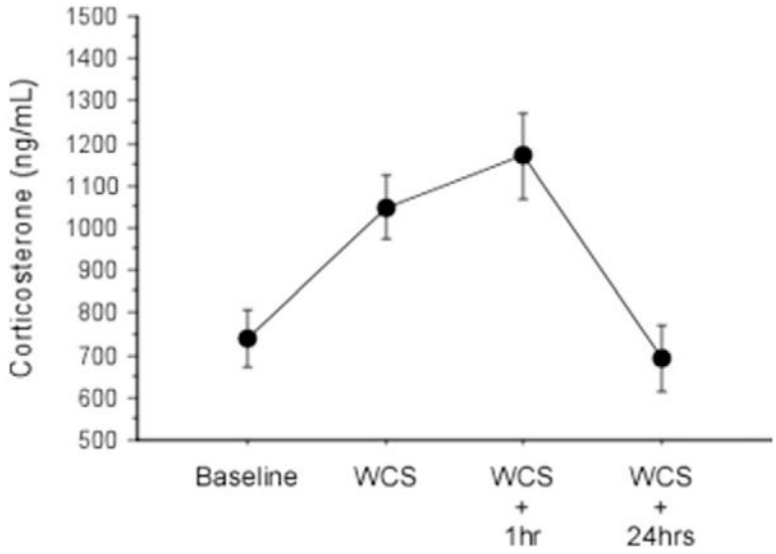

Fig. 7.

Timecourse of CORT in response to the wet cage stressor (WCS). Plasma CORT concentration is shown at baseline, immediately following the WCS, 1 h following the WCS, and 24 h following the WCS.