Hypothalamic Oxytocin Mediates Social Buffering of the Stress Response

Supplement 1

Supplemental Methods & Materials

Voles

Subjects were captive-bred female prairie voles (*M. ochrogaster*) descended from populations in southern Illinois. Voles were weaned on postnatal day 21 and housed with a same-sex conspecific in Plexiglas cages (29 L × 18 W × 13 H cm) containing cedar chip bedding with food and water *ad libitum*. Colony rooms were maintained on a 14L:10D photoperiod (lights on at 0700 hr) and at a temperature range of 21 ± 1°C. All female subjects that were used in this study were sexually naïve, prior to pairing with a male cagemate. Female prairie voles were pair-housed with an unrelated, vasectomized male for two weeks, a sufficient time period that reliably leads to pair-bond formation in prairie voles (1). Experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at Florida State University.

Immobilization Stress

Physical restraint has been used in a number of different rodent animal models to induce stress, including prairie voles (2). On the day of testing, females were exposed to an acute immobilization stress paradigm that our lab has recently developed to elicit a behavioral and physiological stress response in female prairie voles. Subjects were exposed to 1 hr immobilization stress in restraint tubes constructed from 50 mL centrifuge tubes (115 L x 29 OD mm) with the top third of the tube removed to allow for adjusting the diameter for the size of the animal and unrestricted respiration. Prior to placement into these restraint tubes, the fore- and

hindlimbs of females were bound with double-sided hook-and-loop fastener straps. The animals were placed into the restraint tubes, then a second set of straps was placed around the outside of the tube that covered over the body, restricting the diameter of the tube if needed to compensate for the size of the animal, and head of the animal, leading to complete physical immobilization.

Behavioral Measures

The elevated plus maze test was conducted for 5 min using an established method (2). Briefly, subjects were placed in the center facing an open arm and recorded with a video/computer system. Several behaviors were quantified by a trained observer blind to the treatment using *J-Watcher V1.0* (Macquarie University and UCLA; http://www.jwatcher.ucla.edu/) for anxiety-like responses (latency to enter the open arm, percentage of time spent on the open arms vs. total arm time, and percentage of open arm entries vs. total arm entries) and locomotor activity (total arm entries). In a second cohort, the occurrence and duration of female stress-related behaviors and female and male social behaviors were recorded for 1 hr during baseline conditions while in the home cage and 1 hr during the recovery period after the female was immobilized. Stress-related behaviors included rearing, autogrooming, and route tracing, while social behaviors included olfactory investigating, initiating contact with (a.k.a., approaching), following, and allogrooming the partner. Male sexual behavior (i.e., mounting) was also recorded. These behaviors were quantified by a trained observer blind to the treatment using *J-Watcher V1.0*. The occurrences of these behaviors were divided into 30 min periods, and a raw change score was calculated for each behavior (post-stress values minus pre-stress values).

Blood Preparation and Corticosterone Radioimmunoassay

Trunk blood (~400 µl) was collected following rapid decapitation into microcentrifuge vials containing 20 µl EDTA. The vials were inverted and immediately placed in ice. The entire blood collection procedure until chilling did not exceed 2 min. Blood was centrifuged at 6000 rpm for 15 min at 4°C, then plasma was aspirated and centrifuged at 6000 rpm for 10 min at 4°C. Plasma corticosterone (1:1000) was measured (in duplicates) in 10 µl plasma samples, using a commercially available kit (Diagnostic Products Corp., Los Angeles, CA) previously used and validated in prairie voles (2, 3). The detecting limit of the radioimmunoassay kit was 7.7 ng/mL for corticosterone. The intra-assay coefficient of variation (CV) was 2.85%, and all samples were measured in a single assay.

Brain Tissue Preparation and Protein Extraction

After rapid decapitation, brains were collected and then frozen at -80°C. Brains were sectioned coronally at 300 µm and thaw-mounted onto Superfrost Plus slides. A number of brain regions were selected for analysis which have been implicated in the regulation of stress response or social interactions and express receptors for oxytocin, vasopressin, or corticotrophin-releasing hormone (CRH) systems, including the paraventricular nucleus (PVN), nucleus accumbens (NAcc), central amygdala (CeA), and medial amygdala (MeA) (1, 4-6). These brain regions were identified using the Paxinos and Watson rat brain atlas (PVN: Plates 42–49, NAcc: Plates 9–11, MeA: Plates 48–63, and CeA: Plates 48–58) (7). Bilateral tissue punches of a 1 mm diameter (0.5 mm for PVN) were taken from three sections of each brain region and stored at −80°C until processed. Brain tissue punches were lysed through sonication and centrifuged in a RIPA buffer (10 mM Trizma Base, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100,

1% sodium deoxycholate, pH 7.4) containing 1:100 protease inhibitor and 1:100 phosphatase inhibitor. The total protein concentration of each sample (1 µl) was assayed using a DC protein assay (BioRad Labs; Hercules, CA) and read by Gen 5.1 and a Biotek microplate reader.

Western Blots for Vasopressin, CRH, Oxytocin Receptor, and V1a Receptor

Protein extract from the brain tissue samples (20 µg of total protein) was electrophoresed on precast 10–20% SDS gradient gels. After transferring the protein from the gel to a PVDF membrane, the membrane was washed using 1X Tris-buffered saline with 0.1% Tween-20 (TBS-T) and then blocked in 5% w/v milk for 1 hr at room temperature (~23°C). After the general preparation of the membrane, the membrane was incubated with an antibody appropriate for vasopressin, CRH, oxytocin receptor, or V1a receptor diluted in a blocking buffer at 4°C for various lengths of time (Table S3, selectivity of the anti-oxytocin receptor antibody was validated in Figure S1). Subsequently, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody that labels the primary antibody at 25°C for 1 hr, and bands were visualized by enhanced chemiluminescence on x-ray film. After the visualization step for each marker, the membrane was incubated in a stripping solution and then blocked in 5% w/v milk for 1 hr at room temperature before repeating the process for the next marker. The optical densities for vasopressin, CRH, oxytocin receptor, and V1a receptor labeling on x-ray film for each sample were quantified using a computerized image program (Image J 1.44p, Rasband, WS, NIH; http://imagej.nih.gov/ij/). These values were normalized for band intensities using the optical density of β-actin for that sample and standardized by the average normalized optical density of that marker for the handle controls.

Oxytocin Enzyme Immunoassay

Oxytocin concentrations were measured (in duplicate) in the tissue punch protein extracts from the PVN, supraoptic nucleus of the hypothalamus, NAcc, CeA, and MeA using an enzyme immunoassay kit (Assay Designs INC., Ann Arbor, MI). Non-extracted samples were diluted at 1:20 for oxytocin (5 µl of protein extract) and assayed according to kit instructions. The total oxytocin content for each sample was corrected by the total protein content of that sample. The detecting limit of the enzyme immunoassay kit was 11.7 pg/mL. The intra-assay and inter-assay CV was 2.56% and 3.70%, respectively. In addition, oxytocin concentrations were determined from the dialysate samples using the sample protocol. The intra-assay and inter-assay CV was 1.32% and 4.17%, respectively. The oxytocin concentration was not detectable in the NAcc, CeA, or MeA.

Intra-PVN Administration of Oxytocin and Oxytocin Receptor Antagonist

After 7 d of pairing with a male cagemate, female voles were anesthetized and then stereotaxically implanted with 26-gauge guide cannulae (Plastics One) aimed at the PVN (nose bar; AP, -0.74 mm; ML, ± 1.5 mm; DV, 5.0 mm; angle, ± 15°). The voles were allowed 7 d of post-operative recovery. On the day of testing, voles were exposed to the 60-min immobilization stressor. Following this stressor, oxytocin (10 ng or 100 ng), or a selective oxytocin receptor antagonist (10 ng or 100 ng; des-Gly-NH2,d(CH2)5[Tyr(Me)2,Thr4]OVT, kindly provided by Dr. Maurice Manning, Toledo, OH) was dissolved in artificial cerebrospinal fluid (aCSF; a solution isotonic for potassium (2.8 mM KCl), sodium (144 mM NaCl), magnesium (0.9 mM $MgCl_2$), and calcium (1.2mM $CaCl_2$)) and delivered to immobilized voles by infusion in a 200 nl volume over 60 s, with the microinjection needle held in place for 30 s after injection to assure

infusion. Vehicle-treated voles were injected with 200 nl aCSF. At the end of the experiment, brains were collected for histological verification of cannulae placement.

PVN Microdialysis

Microdialysis probe construction and dialysate collection were previously described (8). Briefly, the active area of the dialysis membrane was 1.0 mm and had a molecular weight cutoff of 18 kDa. Probes were perfused continuously at 1.0 µL/min with an aCSF solution using a glass Hamilton syringe connected to an automatic micropump (World Precision Instruments). Voles were anesthetized and then stereotaxically implanted with a microdialysis probe aimed at the PVN (nose bar; AP, -0.7 mm; ML, 0.15 mm; DV, 6.35 mm). Voles were given 24 h to recover, then dialysate samples were collected every 15 min for eight consecutive samples before immobilization stress (only samples 5-8 were used as baseline as the first four were collected to acclimate the vole to the collection process), during the 1 hr immobilization stress (samples 9-12), and during the 1 hr post-immobilization recovery period (samples 13-16) into vials containing 5 µl 0.1 N HCl. Samples were immediately frozen on dry ice. Oxytocin concentrations were too low for detection in many samples during pilot experiments. In order to improve detection of oxytocin in these samples, we pooled the final four baseline samples (samples 5-8) and the first and last two samples during the stress period (samples 9-10 and 11-12) and the recovery period (samples 13-14 and 15-16). This resulted in one 1-hr baseline sample and two 30-min samples during the stress and recovery periods each. After collection was complete, all samples were stored at -80°C until processed via the oxytocin enzyme immunoassay. Brains were collected for histological verification of probe placement.

Data Analysis

One-way analyses of variance (ANOVAs) were used for experiments that compared effects of recovery conditions. Mixed-model ANOVAs were used to compare effects of 1) recovery conditions, 2) recovery conditions and time, and 3) recovery conditions and drug treatment, and the Student-Newman-Keuls *post-hoc* test was used if any main effects or interactions reached statistical significance ($p < 0.05$). One-sample and paired *t*-tests were used to evaluate the changes in stress-related and social behaviors (raw change scores were calculated for each individual by subtracting pre-stress values from post-stress values), respectively. Data were analyzed using IBM SPSS Statistics 19 (SPSS, Inc., an IBM Company) and were expressed as mean ± SEM.

Table S1. Social support continued to affect female stress behavior but not female or male social behavior 30 min after immobilization.

		Female Stress-Related Behavior			
Groups	***n***	**Stress Index**	**Rearing**	**Self-grooming**	**Route Tracing**
Alone	6				
Frequency		10.58 ± 6.45	4.42 ± 4.59	5.08 ± 2.68	1.08 ± 0.46
Duration		32.64 ± 37.66	5.70 ± 5.91	4.80 ± 28.30	22.84 ± 11.44
Partner	7				
Frequency		-6.64 ± 4.17	-1.29 ± 1.50	-5.79 ± 2.12*	0.36 ± 0.32
Duration		-79.43 ± 39.11	-3.70 ± 4.70	-86.21 ± 28.18*	4.35 ± 3.81

		Female Social Behavior			
Groups	***n***	**Olf. Investigation**	**Contact**	**Follow**	**Allogroom**
Partner	7				
Frequency		-0.57 ± 0.70	-4.36 ± 5.61	0.0 ± 0.0	-0.93 ± 0.63
Duration		-4.08 ± 3.30	-227.10 ± 248.23	0.0 ± 0.0	-12.38 ± 10.89

		Male Social Behavior			
Groups	***n***	**Olf. Investigation**	**Contact**	**Follow**	**Allogroom**
Partner	7				
Frequency		-3.79 ± 3.93	-2.71 ± 2.54	0.07 ± 0.17	1.79 ± 1.93
Duration		-15.26 ± 18.46	49.79 ± 116.71	0.10 ± 0.34	96.23 ± 49.53

Values represent a raw change score for each behavior (post-stress minus pre-stress values) reported as means and standard errors. Durations are measured in seconds. Groups include immobilized females recovering alone (Alone) or with a pair-bonded partner (Partner). Asterisks indicate significant difference between pre- and post-stress values ($p < 0.05$). Olf., olfactory.

Table S2. Stress and social support had no effect on neuropeptide and receptor content outside of the paraventricular nucleus.

Brain Region	Marker	HAN	SC	Alone	Partner	*p-value*
NAcc	CRH	1.00 ± 0.20	0.98 ± 0.16	0.90 ± 0.11	1.11 ± 0.20	0.85
	AVP	1.00 ± 0.09	1.09 ± 0.16	1.08 ± 0.19	1.40 ± 0.39	0.64
	V1aR	1.00 ± 0.32	0.94 ± 0.30	0.89 ± 0.30	1.20 ± 0.56	0.94
	OTR	1.00 ± 0.36	0.86 ± 0.23	1.02 ± 0.32	1.14 ± 0.51	0.96
MeA	CRH	1.00 ± 0.42	0.99 ± 0.36	1.15 ± 0.11	1.36 ± 0.32	0.81
	AVP	1.00 ± 0.19	0.49 ± 0.09	1.22 ± 0.25	0.83 ± 0.16	0.11
	V1aR	1.00 ± 0.19	1.42 ± 0.88	1.14 ± 0.23	0.84 ± 0.18	0.78
	OTR	1.00 ± 0.21	1.54 ± 0.97	1.32 ± 0.36	1.03 ± 0.24	0.83
CeA	CRH	1.00 ± 0.27	0.62 ± 0.16	0.73 ± 0.17	0.86 ± 0.23	0.64
	AVP	1.00 ± 0.24	0.73 ± 0.10	0.69 ± 0.04	0.84 ± 0.19	0.51
	V1aR	1.00 ± 0.10	1.29 ± 0.29	1.13 ± 0.14	1.65 ± 0.23	0.16
	OTR	1.00 ± 0.27	0.87 ± 0.19	0.71 ± 0.13	0.95 ± 0.20	0.71
SON	OT	18.46 ± 4.71	17.11 ± 1.38	16.70 ± 5.80	22.75 ± 3.91	0.62

Means and standard errors are provided for each protein marker. Values for corticotrophin-releasing hormone (CRH), vasopressin (AVP), vasopressin 1a receptor (V1aR), and oxytocin receptor (OTR) reflect the optical density as measured via Western blotting (normalized to actin and standardized to control values). Values for oxytocin (OT) in the supraoptic nucleus of the hypothalamus (SON) are provided in ng/mL as measured via enzyme-linked immunosorbent assay (ELISA). Groups include handle controls (HAN), social controls (SC), and immobilization stress followed by recovery alone (Alone) or with a male partner (Partner). OT was not detectable in the nucleus accumbens (NAcc), medial amygdala (MeA), or central amygdala (CeA).

Table S3. Antibodies for Western blotting in the prairie vole brain

Primary Antibody	Dilution	Incubation	MW	Secondary Antibody
Mouse anti-Actin [Millipore; MAB1501]	1:10K	18-24 hr	45	Bovine anti-Mouse IgG-HRP [Santa Cruz; sc-2371]
Rabbit anti-AVP [Millipore; AB1565]	1:4K	18-24 hr	17	Goat anti-Rabbit IgG-HRP [Santa Cruz; sc-2030]
Rabbit anti-V1aR [Enzo; ADI-905-811]	1:100	42-48 hr	48	Goat anti-Rabbit IgG-HRP [Santa Cruz; sc-2030]
Goat anti-CRF [Santa Cruz; sc-1759]	1:500	18-24 hr	22	Donkey anti-Goat IgG-HRP [Santa Cruz; sc-2033]
Goat anti-OTR [Santa Cruz; sc-8102]	1:1000	42-48 hr	63	Donkey anti-Goat IgG-HRP [Santa Cruz; sc-2033]

Molecular weight (MW) is shown in kDa. All primary antibodies were incubated in 4°C. The dilution and incubation for all secondary antibodies were 1:10K for 1 hr at room temperature (23°C).

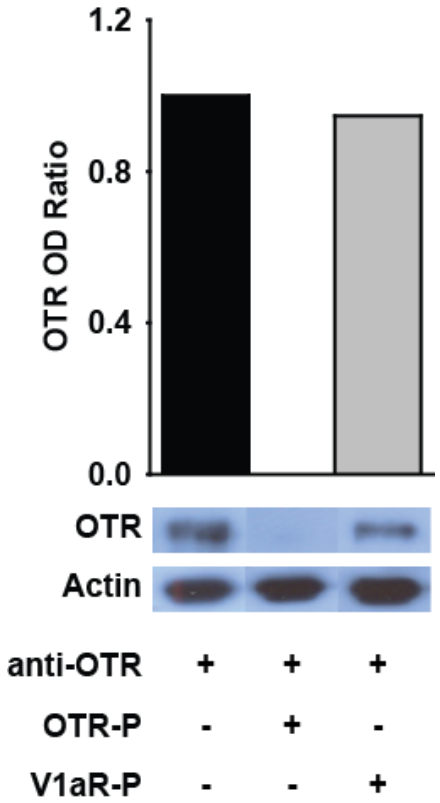

Figure S1. Validation of the Anti-Oxytocin Receptor (anti-OTR) Antibody. Samples derived from pooling together the protein extract from the paraventricular nucleus of the hypothalamus of several handled control females. Western blotting was completed as described (see Supplemental Methods & Materials); however, the anti-OTR antibody was pre-incubated at 4°C for 48 hr in (Lane 1) buffer only, (Lane 2) buffer + 1:200 OTR-peptide (Santa Cruz; sc-8102 P), or (Lane 3) buffer + 1:200 V1aR-peptide (sc-18096 P). No band was detected when the anti-OTR antibody was pre-incubated in OTR-peptide, but bands were detected if the anti-OTR antibody was only pre-incubated in buffer or in V1aR-peptide. Thus, the anti-OTR antibody was selectively detecting OTR and not cross-reacting with V1aR. Data were normalized for band intensities using the optical density (OD) of β-actin for that sample and standardized by the normalized OD of the sample incubated with the anti-OTR antibody only.

Supplemental References

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