Sex-Specific Effects of Stress on Oxytocin Neurons Correspond With Responses to Intranasal Oxytocin

Supplemental Information

Supplementary Methods and Materials

Animals

California mice (*Peromyscus californicus*) were raised in our vivarium at UC Davis. Mice were ear punched for identification and maintained in clear polypropylene cages provided with Carefresh or Sanichip bedding and cotton nestlets. Mice were provided with food (Harlan Teklad 2016) and water *ad libitum*. Mice were housed on a photoschedule of 16L:8D (lights off 1400 PST). All mice were 3-6 month old adults and housed in cages of 2-3 same sex individuals. Behavioral assays were conducted during lights out under dim red light (3 lux). At the end of each experiment, mice were anesthetized with isoflurane gas (Minirad Inc., Bethlehem, PA, USA) and then rapidly decapitated. Brains were rapidly removed and either fixed overnight in 5% acrolein (Sigma, St. Louis MO, USA) in 0.1 M phosphate buffered saline (PBS) at 4°C for immunohistochemistry or snap frozen on dry ice for mRNA analysis. Estrous stage was assessed for females by vaginal lavage just after decapitation. This procedure significantly impacts behavior (1), so it was only done after euthanasia. All experiments received approval from the UC Davis Institutional Animal Care and Use Committee and mice were treated in accordance recommendations of the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

Immunohistochemistry

OT/c-fos Immunostaining Procedure: Brains were sliced at 40 mm and sections were then washed two times in PBS. Sections were incubated for 10 min in 0.1M sodium borohydride in PBS and then blocked in PBS containing 10% normal donkey serum (NDS). Sections were incubated overnight incubation at 4°C in rabbit anti-c-fos (1:2500, PC38T, EMD Millipore, Billerica, MA) and made up in antibody diluent [PBS with 0.5% Triton X (Tx), and 2% NDS]. Tissue was washed three times for 5 min each in PBS on the following day and then transferred to a solution containing Alexa Fluor 555 conjugated-donkey anti-rabbit IgG (1:500, #A-31572, Invitrogen, Carlsbad, CA) for 2 hours at room temperature. Following three 5-min washes in PBS tissue was incubated for 48 h in a mouse anti-oxytocin monoclonal antibody (1:2000, clone 4G11 MAB5296, Millipore). After the incubation, tissue was subjected to three 5-min washes in PBS and then incubated 2 h at room temperature in antibody diluent containing a biotin-conjugated donkey anti-mouse secondary (1:250, #715-065-150, Jackson ImmunoResearch, West Grove, PA). After three 5 min-washes in PBS sections were incubated for 30 min in Alexa Fluor 350-conjugated streptavidin (1:250, #S-11249, Invitrogen) in PBS-Tx. Tissue was then washed in PBS for 5 min, then in fresh PBS for 10 min, and then spent 30 min in PBS with 0.1% Tx before a final wash in PBS without detergent for 5 min. Sections were mounted onto Superfrost plus slides (Fisher, Pittsburgh, PA, USA). Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA) was added prior to coverslipping. We previously validated the OT antibody for use in California mice (2).

Immunohistochemical Analysis: Sections were chosen for staining at 120 mm intervals starting at approximately bregma 0.14 (BNST) and going to bregma -1.46. Slides were photographed under a monochromatic Axiocam MRm camera (Carl Zeiss Meditec) through a Zeiss Axioimager (Carl Zeiss Meditec, Inc., Dublin, CA). ImageJ software (NIH, Bethesda, MD) was used to generate and place a box in each area of interest. The box dimensions were 0.35 X 0.43 mm for PVN, 0.26 X 0.28 for BNSTpm, 0.52 X 0.36 mm for BNSTmv, and 0.36 X 0.36 mm for AH and SON. The number of OT-ir neurons, c-fos-ir cells and colocalizations between the two were manually counted by a sex and treatment blind observer.

Enzyme-Linked Immuoassays for OT and Estradiol

Plasma OT concentrations were assessed using an enzyme immunoassay kit (Enzo Life Science, Farmingdale, NY, USA) as previously described (3). The sensitivity of the assay is 3.4 pg/mL. The intra-assay coefficient of variation was 5.4%. Plasma estradiol concentrations were assessed using an enzyme immunoassay kit (Caymen Chemical, Ann Arbor, MI, USA) as previously described (1). The sensitivity of the assay was 6.6 pg/mL and the intra-assay coefficient of variation was 11.1%. Data were log transformed for statistical analysis.

Real Time PCR

RNA was extracted from BNST and PVN punch samples using RNAqueous kits (Life Technologies) and reverse transcribed using iScript (BioRad). Real-time PCR was conducted using SYBR green chemistry with an Applied Biosystems ViiA7 instrument.

Specific forward (CTGCGACCCTGAGTCTGC) and reverse primers (GGAGTGAAGGGTGAGCTCT) were designed to amplify the California mouse *Oxt* mRNA (Genbank accession: JX977028). We also measured *Avp* (Genbank accession: JX977025.1; F primer AGTGTCGCGAGGGTTTTC, R primer GGGCTTGGCAGAATCCAC) and *Crh* (Genbank accession: XM_006974676.1; F primer CGGCCATCTCTCTGGATCTC, R primer CTTCACTAACTGGTCTGCCTTGACT) mRNA. Gene expression was normalized to b-2 microglobulin (Genbank accession: XM006995122.1; F primer TCTAGTGGGAGGTCCTGTGG, R primer TGCGTTAGACCAGCAGAAGG) using the delta-delta Ct method as previously described (2). These primers were tested with a standard curve of serially diluted cDNA obtains from California mouse hypothalamus (1:1-1:10K). We used these reactions to calculate the efficiencies of the *Oxt* (98%), *Avp* (100%), *Crh* (92%), and *B2m* (100%) primers. Each reaction produced a single peak in melting curve analyses and when the resulting PCR product for *Oxt* reactions were run on an agarose gel only one band of the predicted size (82 bp) was observed (Fig. S2).

Intranasal Oxytocin Administration

Oxytocin (Bachem) was diluted in sterile saline at concentrations of 0.8 IU/kg or 8.0 IU/kg (4). The low dose was derived from publically available reports on clinical research and is comparable to intranasal administration of 40 IU OT to a 50 kg human. Intranasal OT infusion was performed 5 min before the start of the respective behavior tests. A blunt cannula needle (33 gauge, 2.8 mm length; Plastics One, Roanoke, Virginia) was connected to cannula tubing, and flushed with saline before drawing up

the experimental solution. The tubing was placed on a Hamilton syringe (Bachem). Mice were hand-restrained and 25 µl of solution were gradually infused into the nasal mucosa, alternating between the left and the right nostrils. Following intranasal infusion, mice were returned to the home cage and any cage mates until the start of testing. Administration typically took less than 30 sec and all groups received equal levels of handling.

Behavioral Procedures

Social Defeat: Male and female mice were subjected to 3 episodes of social defeat or handling control on consecutive days during the first 3 h of lights out (1430–1730) under dim red light. Mice assigned to social defeat were placed into a home cage with an aggressive same sex resident for 7 - 10 min or until the focal mouse was exposed to 7-10 offensive attacks while control mice were kept in an empty cage for an equivalent amount of time. This protocol normalizes the intensity of aggression between males and females and does not result in injuries to the focal mice (5).

Social Interaction Testing: Social interaction testing was performed as previously described (5, 6). Mice were tested in a large Plexiglas box (89 × 63 × 60 cm). During the open field phase, each mouse was placed into the empty arena for 3 min and time spent in the center of the arena and total distance traveled were measured using a video tracking system (Any-Maze, Stoelting). In the acclimation phase of testing a small wire cage was placed against a wall of the arena with the focal mouse still inside the testing arena. The video tracking system recorded time spent inside the target zone

(interaction time), which was defined as the area within 8 cm of the cage. During the interaction phase, an unfamiliar same-sex target mouse was inserted into the wire cage and the time the focal mouse occupied the target zone was recorded. We also calculated the social interaction ratio defined as 100 x (interaction time, target present)/(interaction time, target absent) (7, 8).

Resident-Intruder Testing: Each focal mouse was singly-housed 3 days before resident-intruder testing. Testing was performed within the first 3 h of the start of the dark phase. Testing began when a weight-matched virgin same-sex intruder was introduced into the home cage of the focal mouse. The intruder was removed 7 min later. An infrared camera was used to record behaviors, which were scored by an observer blind to treatment and sex. We quantified the time spent freezing, boxing, engaging in ano-genital sniffing of the intruder as well as attempting to escape the home cage and the number of bites (2). A bout of freezing was considered to be when all four paws of the focal mouse were placed against a surface and did not move any part of the head or body for 2 seconds. When a bout of escape occurred the resident stood beside a wall of the home cage and jumped from one side to the next.

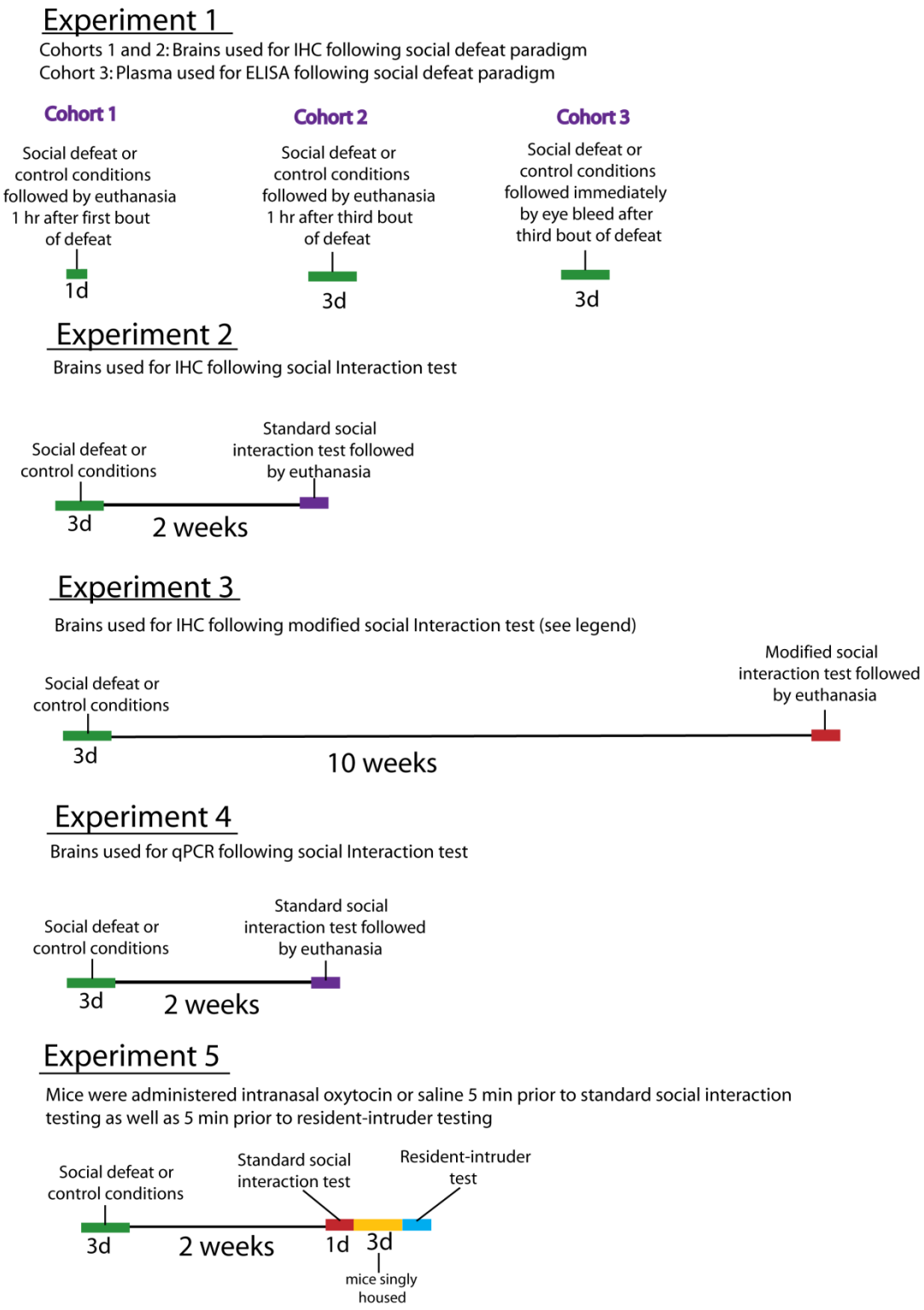

Figure S1. Timelines for experiments 1-5. In experiment 1 there were three cohorts of mice. In Cohort 1 mice were euthanized one hour after one episode of defeat or control conditions. In Cohort 2 mice were euthanized one hour after a third episode of defeat or

control conditions. In Cohort 3 a retro-orbital blood sample from each mouse was collected immediately after a third episode of defeat or control conditions. In experiment 2 each mouse was exposed to control or defeat conditions over three days and then tested in the social interaction test two weeks later. All focal mice were exposed to a target mouse in the final stage of the test and each focal mouse was euthanized 1 h later. In experiment 3 each focal mouse was exposed to control or defeat conditions and then tested as previously described in detail (9). Ten weeks after the last episode of defeat, each mouse was tested in the modified social interaction test. Half of the focal mice were tested with a target mouse (target present) and half were tested with an empty cage (target absent) during the interaction phase. Mice were euthanized 1 h later. In experiment 4 mice each mouse was exposed to control or defeat conditions. Two weeks later each mouse was tested in the social interaction test and euthanized. In experiment 5, behavior experiments were conducted 2 weeks after control or defeat conditions. Each mouse was treated with intranasal saline or oxytocin and then tested in the social interaction test 5 min later. Mice were subsequently individually housed for 3 days. Each mouse received an intranasal infusion of saline or oxytocin 5 min before testing in a resident-intruder test.

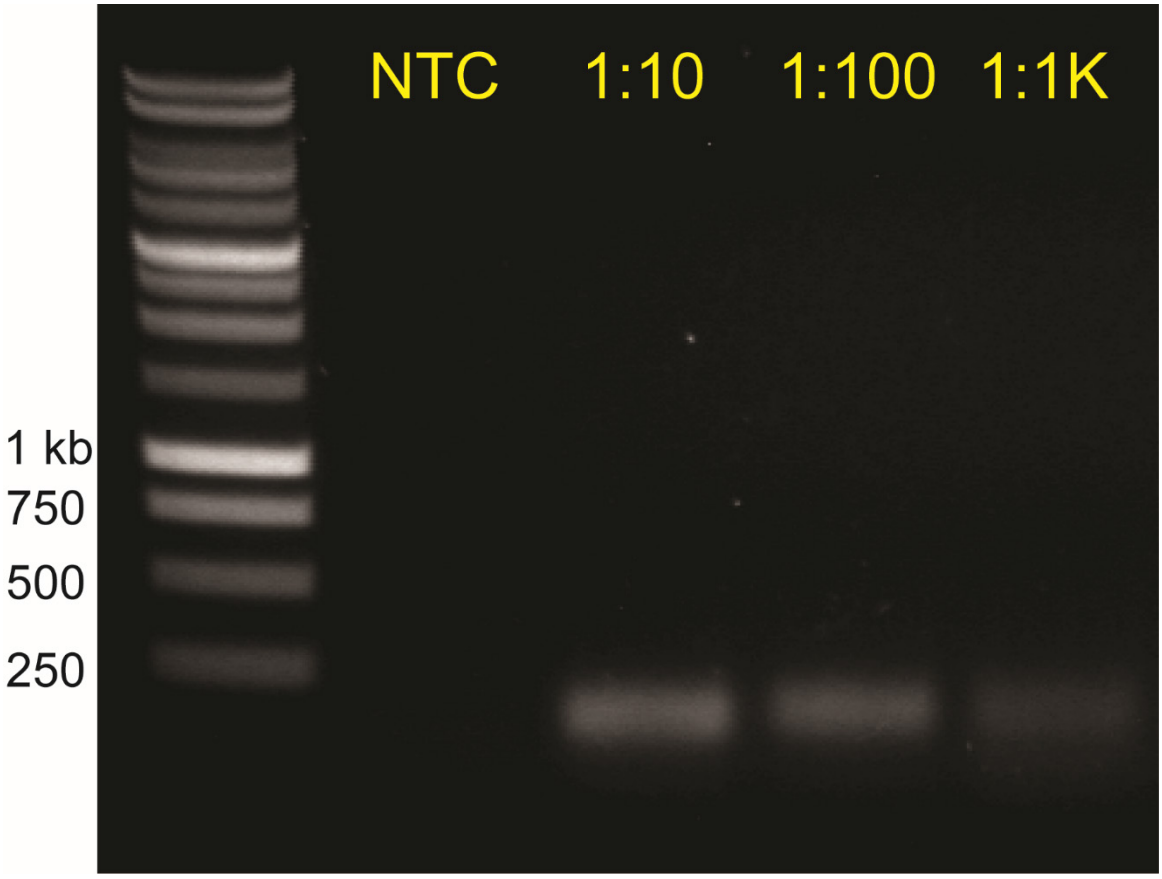

Figure S2. Real-time PCR reactions using primers for *Oxt* mRNA produced a single band of the predicted size (82 bp). Template for reactions consisted of serial dilutions of cDNA obtained from pooled samples of microdissected supraoptic nucleus from California mice. NTC, no template control, 1:10 dilution of cDNA, 1:100 dilution of cDNA, 1:1K dilution of cDNA. Ladder is Promega 1 kb ladder.

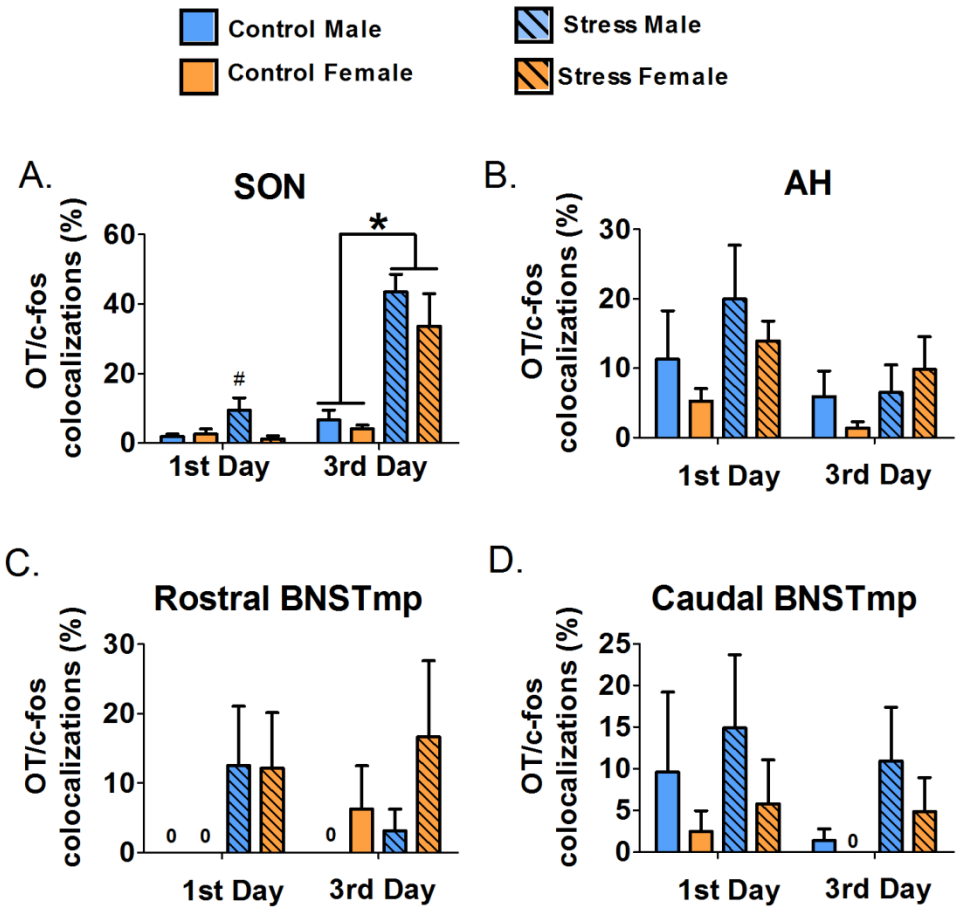

Figure S3. Acute effects of social defeat on oxytocin/c-fos immunoreactivity. Social defeat increased the percentage of OT/c-fos colocalizations in the supraoptic nucleus (SON) after the third day of defeat (**A**) in both sexes and in males after the first day of defeat. There was no effect of defeat on OT/c-fos in the anterior hypothalamus (AH, **B**), or the rostral (**C**) or caudal (**D**) posteromedial bed nucleus of the stria terminalis (BNSTmp). *n* = 6-8 per group. Data are shown as mean ± SE. **p* < 0.01 main effect of defeat, $^{\#}p < 0.05$ effect of defeat in males.

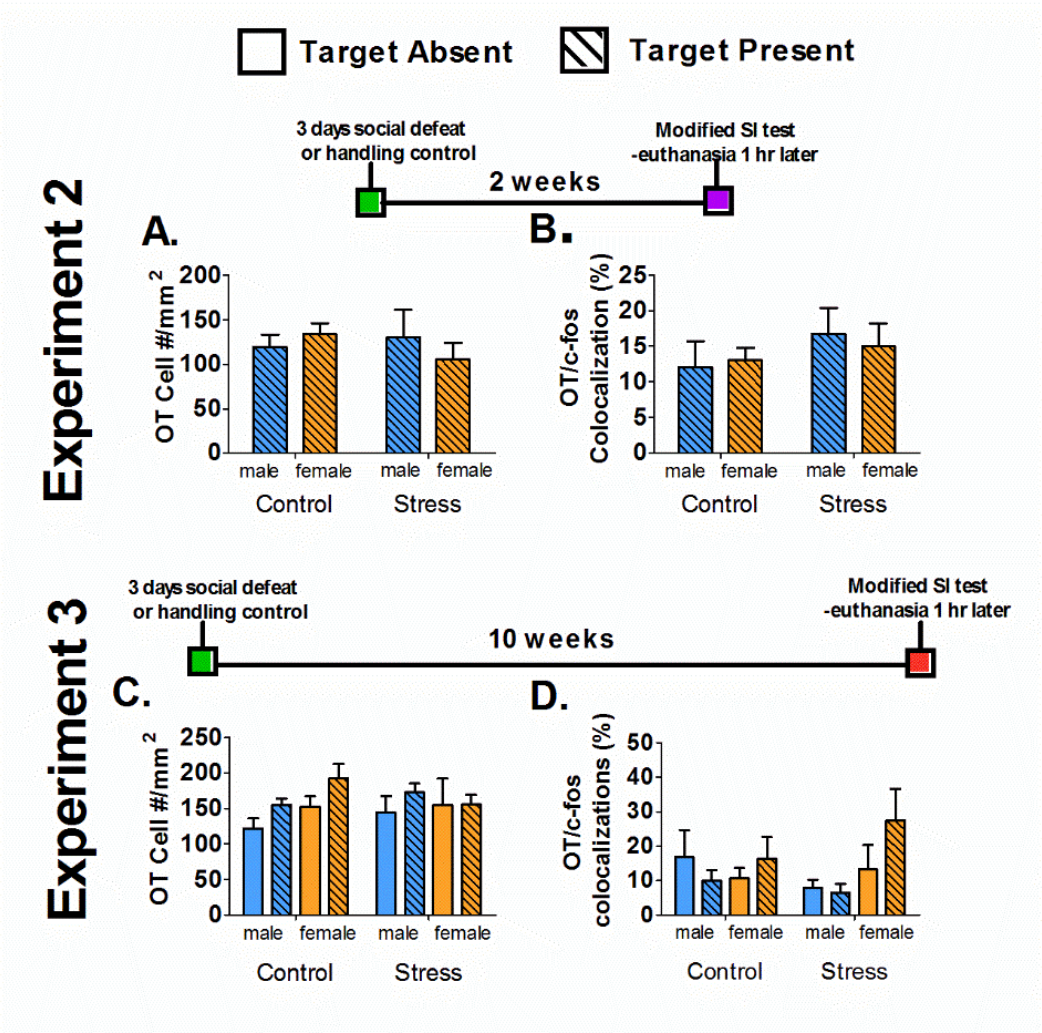

Figure S4. Oxytocin cell counts and percent OT/c-fos data in the caudal paraventricular nucleus (PVN). There were no differences in OT cells (**A**,**C**) or OT/c-fos colocalizations (**B**,**D**) in caudal PVN in either experiment 2 (*n* = 7-8 per group, all mice were tested with the target present) or in experiment 3 (*n* = 4-5 per group, mice were tested with either a target present or absent).

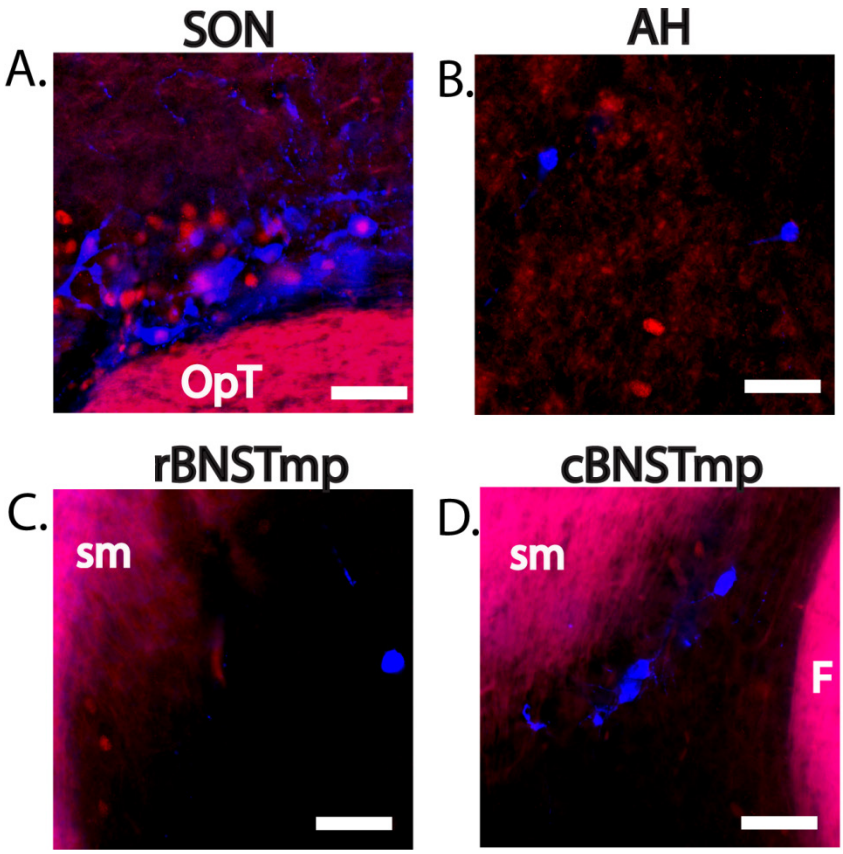

Figure S5. Photomicrographs of oxytocin cells in supraoptic nucleus (SON, A), anterior hypothalamus (AH, B), and rostral (C) and caudal (D) posteromedial bed nucleus of the stria terminalis (BNSTmp). F, fornix; OpT, optic tract; sm, stria medullaris of the thalamus. Scale bars = 50 µm.

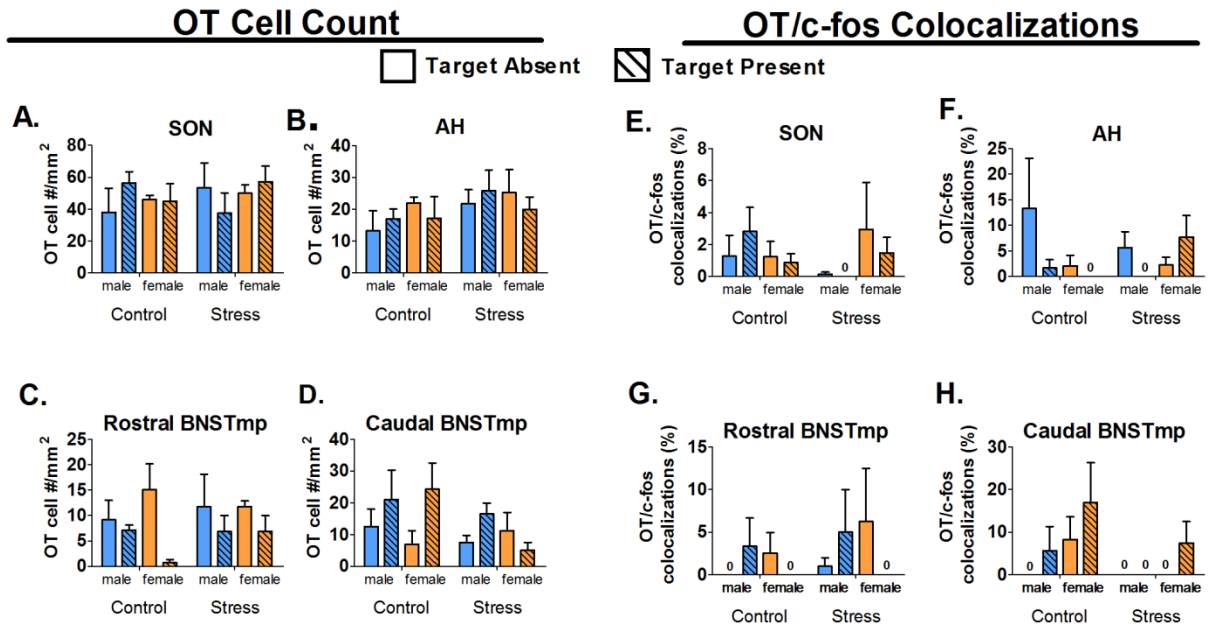

Figure S6. Oxytocin cell counts and percent OT/c-fos data in other hypothalamic brain regions from experiment 3. No differences in OT cell counts or OT/c-fos colocalizations were observed in the supraoptic nucleus (SON; **A**, **E**), anterior hypothalamus (AH; **B**, **F**), rostral posteromedial BNST (BNSTmp; **C**, **G**), or caudal posteromedial BNST (**D**, **H**). *n* = 4-5 per group.

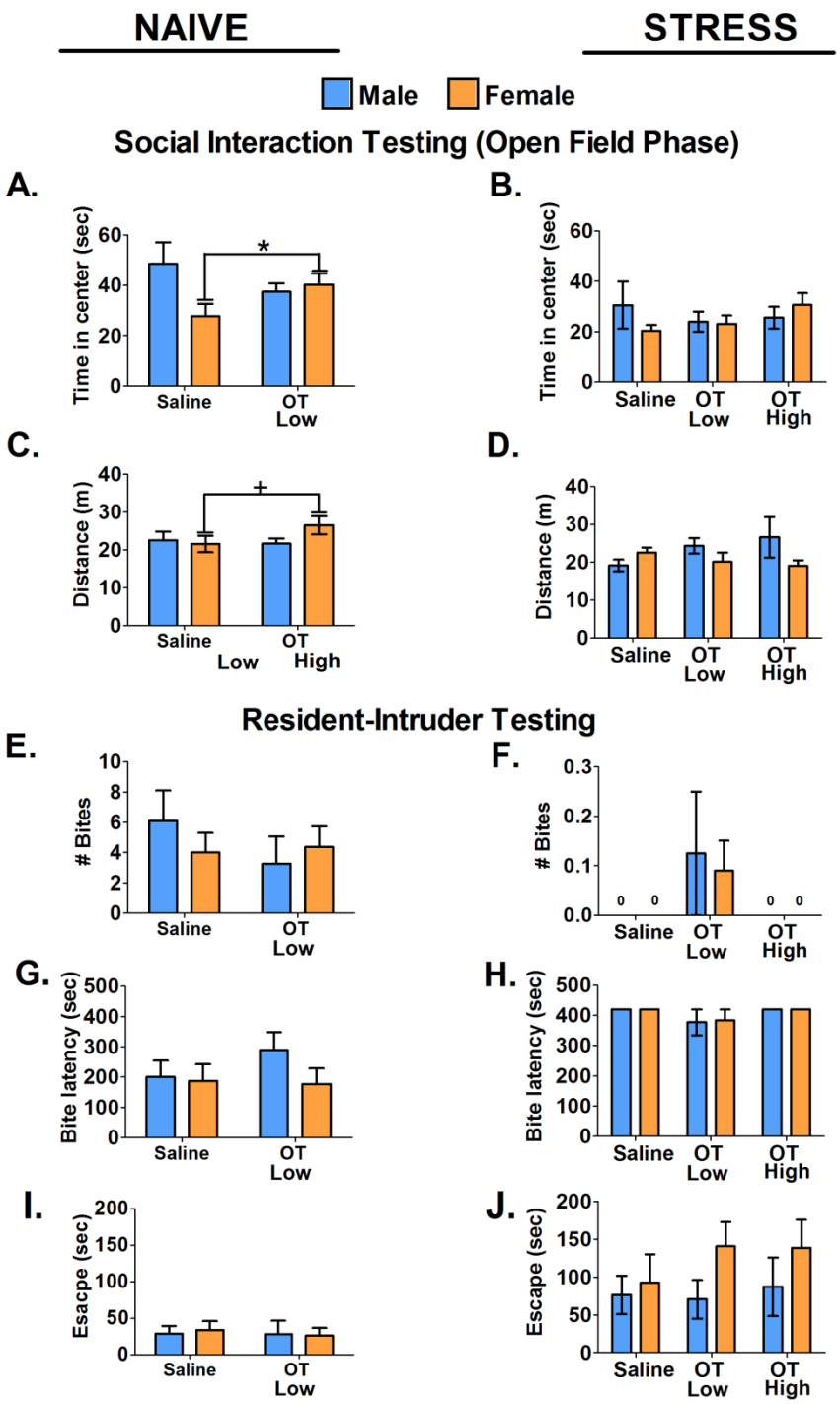

Figure S7. Effects of intranasal OT on behavior in the open field (A-D) and resident-intruder (E-J) tests. In mice naive to defeat the 0.8 IU/kg dose of OT increased time in the center of the open field in females but not males (**A**). No differences were observed in stressed mice (**B**, **D**). In resident-intruder tests intranasal OT had no effect on bites, bite latency, or escape behavior (**E**-**J**). *n* = 11-13 per group. **p* < 0.05, within sex comparisons using Mann-Whitney test.

Table S1. Total cell counts of oxytocin (OT), c-fos, and OT/c-fos positive cells per mm^2 within the medioventral bed nucleus of the stria terminalis (BNSTmv) and the rostral paraventricular nucleus (rPVN) from experiments 2 and 3. Total c-fos counts for experiment 3 were previously reported in Supplementary Table 1 of Steinman *et al.* (2).

		OT	c-fos	OT/c-fos
Exp. 2				
BNSTmv	Control Male	18.7 ± 4.7	151.6 ± .29.9	0 ± 0
	Stress Male	16.8 ± 9.9	217.3 ± 43.7	0.8 ± 2.0
	Control Female	9.1 ± 5.3	206.8 ± 35.5	0 ± 0
	Stress Female	34.7 ± 10.1*	233.7 ± 28.3	3.3 ± 1.4*
rPVN	Control Male	277.1 ± 24.2	90.3 ± 22.7	8.3 ± 3.1
	Stress Male	275.8 ± 15.6	169.5 ± 23.9	26.12 ± 6.8*
	Control Female	315.2 ± 24.7	192.2 ± 31.0	17.2 ± 3.7
	Stress Female	258.1 ± 14.5*	218.9 ± 33.4	30.2 ± 6.0
Exp. 3				
BNSTmv	Control Male; Target Absent	61.5 ± 8.9	171.5 ± 44.1	9.6 ± 4.0
	Stress Male; Target Absent	24.6 ± 13.1#	103.6 ± 22.1	0 ± 0#
	Control Male; Target Present	29.4 ± 8.7	205.1 ± 40.9	2.1 ± 2.1
	Stress Male; Target Present	31.0 ± 10.8	28.3 ± 9.3*	1.1 ± 1.1
	Control Female; Target Absent	29.4 ± 6.0	105.5 ± 21.3	0.5 ± 0.5
	Stress Female; Target Absent	56.8 ± 19.0*	156.9 ± 22.5	8.0 ± 1.7*
	Control Female; Target Present	31.6 ± 11.8	141.6 ± 39.2	2.7 ± 1.3
	Stress Female; Target Present	58.8 ± 13.9*	141.0 ± 40.9	11.8 ± 6.3*
rPVN	Control Male; Target Absent	119.7 ± 9.2	438.0 ± 65.1	19.0 ± 5.4
	Stress Male; Target Absent	119.3 ± 22.2	333.9 ± 71.7	16.5 ± 8.6
	Control Male; Target Present	136.1 ± 16.7	259.7 ± 91.3	31.6 ± 13.7
	Stress Male; Target Present	133.2 ± 10.7	201.7 ± 74.9	15.8 ± 6.0
	Control Female; Target Absent	134.4 ± 25.4	394.7 ± 60.1	26.5 ± 8.0
	Stress Female; Target Absent	129.4 ± 21.2	507.3 ± 22.7	17.3 ± 5.3
	Control Female; Target Present	140.0 ± 25.4	425.0 ± 66.7	32.9 ± 8.8
	Stress Female; Target Present	70.6 ± 10.3#	477.0 ± 112.0	27.7 ± 7.4

*$p < 0.05$ effect of stress within sex.
#$p < 0.05$ effect of target within sex.

Table S2. Estradiol analysis of covariance of cell counts from Experiment 2

BNSTmv				
	Estradiol	**Stress**	**Cage**	**Stress x Cage**
Oxytocin	$F_{(1,14)}$ = 0.04, p = 0.85	$F_{(1,14)}$ = 4.07, p = 0.06	$F_{(1,14)}$ = 0.01, p = 0.93	$F_{(1,14)}$ = 0.00, p = 0.99
Oxytocin/c-fos	**$F_{(1,14)}$ = 5.58, p = 0.03**	**$F_{(1,14)}$ = 9.46, p < 0.01**	$F_{(1,14)}$ = 0.28, p = 0.61	$F_{(1,14)}$ = 0.95, p = 0.35

Table S3. Real-time PCR results for BNST and PVN gene expression. Gene expression data normalized to *B2m* and expressed as percent control male.

	BNST	**PVN**
	Avp	***Crh***
Control Male	100.0 ± 48.7	100.0 ± .40.7
Stress Male	33.0 ± 12.7	55.6 ± 24.1
Control Female	62.6 ± 38.9	66.1 ± 27.0
Stress Female	25.6 ± 15.4	114.9 ± 30.5

Supplemental References

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