Enhanced corticosterone concentrations and attenuated Fos expression in the medial amygdala of female oxytocin knockout mice exposed to psychogenic stress

Rose C. Mantella, Regis R. Vollmer, Linda Rinaman, Xia Li, and Janet A. Amico

Departments of \textsuperscript{1}Pharmaceutical Sciences, \textsuperscript{2}Medicine, and \textsuperscript{3}Neuroscience,

University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Submitted 8 June 2004; accepted in final form 12 August 2004

Mantella, Rose C., Regis R. Vollmer, Linda Rinaman, Xia Li, and Janet A. Amico. Enhanced corticosterone concentrations and attenuated Fos expression in the medial amygdala of female oxytocin knockout mice exposed to psychogenic stress. Am J Physiol Regul Integr Comp Physiol 287: R1494–R1504, 2004. First published August 19, 2004; doi:10.1152/ajpregu.00387.2004.—Centrally released oxytocin (OT) is involved in processes that include maternal (50) and affiliative behavior (13, 71), lordosis (6), ingestion of food (64) and sodium chloride-containing solutions (1, 3, 52, 60, 63), social memory (22, 23), and grooming (18). In addition, central OT is believed to lessen stress response. Indeed, recent reports suggest that the MeA is connected to the medial parvocellular paraventricular nucleus of the hypothalamus (PVN; the site of neurons that release CRH) either directly (54, 58, 62) or via a neural circuit that involves synapses in the bed nucleus of the stria terminalis (BNST) and the medial preoptic area of the hypothalamus (mPOA) (11, 29, 30, 51). OT-immunoreactive neurons originating in the PVN project to the BNST (15, 31), and OT-immunoreactive fibers have been identified in the amygdala (15, 31). In addition, the projections of OT neurons correspond to the location of OT receptors in the limbic system and include the BNST, central (CeA) and medial nuclei of the amygdala (MeA), septum, and hippocampus (34). OT and its receptor are located in brain areas that modulate the hypothalamic pituitary adrenal (HPA) axis response to psychogenic stress. The MeA in rats is connected to the medial parvocellular PVN via relays to the BNST and mPOA. Stimulation of the MeA activates the HPA axis, resulting in corticosterone release (19) and excitotoxic lesions of the MeA result in the suppression of HPA axis activation in response to psychogenic stress in the rat (16). Furthermore, exposure to restraint stress activates the MeA as well as OT neurons within the PVN (16). Therefore, it is possible that OT modulates activation of the MeA during the stress response. Indeed, recent reports suggest that the MeA is a target for OT actions in mice (22, 40, 46) and in rats (69).

To further assess the role of the central oxytocinergic system in the stress response, we measured plasma corticosterone concentrations in female OT knockout and wild-type mice under control conditions and after exposure to shaker stress, a predominantly psychogenic stress. Female mice were used in this study because OT mRNA expression (49, 57), OT receptor binding (75), and stress- and anxiety-related effects of OT are facilitated by estrogen (39, 40). The role of OT in the stress response of female mice (8, 38) and rats (69, 70) has been
examined using several psychogenic stressors. If central OT attenuates the HPA axis response to stress, we hypothesize that OT knockout mice will have higher concentrations of corticosterone than wild-type mice. We also used an immunohistochemical approach to localize stressor-induced neural expression of the immediate-early gene product Fos together with markers for OT, AVP, or CRH to identify brain circuits that are activated in mice after exposure to shaker stress and to determine the chemical phenotypes of stress-activated neurons.

MATERIALS AND METHODS

Animals

Female wild-type (OT+/+) and OT knockout (OT−/−) mice, which do not synthesize or release OT (6–9 mo of age; C57BL/6 background strain), were used for this study. OT−/− mice were generated by Dr. Scott Young, National Institute of Mental Health (Bethesda, MD) (76). The original breeding pairs that were used to establish the colony in Pittsburgh were purchased from Jackson Laboratories (Bar Harbor, ME). Mice used for these studies were from the F4 and F5 generation. OT+/+ male and OT+/+/ female mice were mated to produce OT+/+ mice. OT−/− mice were the offspring of male OT−/− and heterozygous (OT+/−) female mice (3). Groups of four or five animals per cage were maintained on a 12:12-h light/dark cycle (lights on at 0700) in temperature controlled, virus-free quarters of the University of Pittsburgh Animals Facility. Food and water were provided ad libitum. DNA was extracted from a tail sample and analyzed by polymerase chain reaction (PCR) (3, 76) to identify the genotype of each mouse. Pairs of primers for PCR that detected either the wild-type allele (OT) or the mutant allele (neomycin resistance cassette) were synthesized at the University of Pittsburgh Sequence facility. Using the primer pairs separately allowed us to genotype each mouse as wild type (expressing the OT gene), OT knockout (expressing the neomycin resistance cassette), or heterozygous (expressing both the wild-type allele and the neomycin resistance cassette). The Institutional Animal Care and Use Committee of the University of Pittsburgh approved all studies.

Experimental Protocols

Female mice were individually housed in standard rodent boxes for 1 wk before and during testing, except for experiment 1A, in which mice were group housed. Studies were conducted between 0800 and 1200, corresponding to the nadir of corticosterone secretion in rodents (14, 77). In each experiment, exposure to the stressor and death took place in separate rooms. On the day of the experiment, mice were tested as paired cohorts according to genotype and treatment group.

Experiment 1A. Corticosterone response to shaker stress in group-housed mice. Shaker stress, originally described as an environmental stressor that would release corticosterone in mice and to determine the time course of corticosterone release after termination of the stress. The duration of shaker stress was chosen based on studies performed in rats showing increased peripheral and central OT release after 10 min of shaker stress (45). To keep environmental stress to a minimum during the experiment, mice were housed, exposed to shaker stress, and killed in different rooms. On the basis of the experimental constraints of performing the study in three rooms and the fact that plasma corticosterone concentrations in rats typically peak 15–30 min after exposure to stress (17), plasma corticosterone concentrations in mice were evaluated at 10 and 30 min after exposure to shaker stress. Group-housed OT+/+ and OT−/− mice were exposed to shaker stress for 10 min and killed 10 (OT+/+ n = 4, OT−/− n = 6) or 30 (OT+/+ n = 4, OT−/− n = 6) min poststress. Control mice (OT+/+ n = 3, OT−/− n = 6), not exposed to shaker stress but maintained the same as mice receiving stress, were killed at the same times. Trunk blood was obtained for measures of corticosterone. Compared with control mice, corticosterone concentrations increased after shaker stress at each time point. Because corticosterone levels were significantly higher in OT−/− mice compared with OT+/+ mice 10 and 30 min after stress, subsequent studies only examined corticosterone levels in mice 10 min after termination of shaker stress.

Experiment 1B. Corticosterone response to shaker stress in individually housed mice. Experiment 1A was repeated in OT+/+ (n = 7) and OT−/− (n = 7) mice that were individually housed for the week before and during the experiment in contrast to the first study in which animals were group housed. Mice were individually housed in an effort to reduce variability in corticosterone levels measured within experimental groups. Mice were subjected to shaker stress for 10 min and killed 10 min after termination of the stressor. Control OT+/+ (n = 8) and OT−/− (n = 8) mice, which were not exposed to shaker stress but were maintained and handled as mice receiving stress, were killed at the same time. Trunk blood was obtained for measures of plasma corticosterone.

Experiment 2. Corticosterone response to shaker stress during the estrous cycle. To determine if the corticosterone response to shaker stress is influenced by the stage of the estrous cycle, OT+/+ and OT−/− mice were killed 10 min after the termination of a 10-min shaker stress at each of the following stages of the estrous cycle: diestrus (OT+/+ n = 6, OT−/− n = 6), proestrus (OT+/+ n = 5, OT−/− n = 8), and estrus (OT+/+ n = 6, OT−/− n = 6). Trunk blood was collected at death to determine plasma corticosterone concentrations. Vaginal cytology was used to predict the stage of the cycle at which mice were to be exposed to shaker stress. Daily vaginal smears were followed for three to four consecutive cycles before experimentation. Only those mice with regular cycles were used in these experiments (94% of the total number of OT+/+ mice and 91% of the total number of OT−/− mice tested).

Experiment 3. Corticosterone response to repeated shaker stress. In rats, repeated exposure to shaker stress has been found to dampen the corticosterone response (27). To determine if repeated exposure to shaker stress would eliminate the genotypic differences in the corticosterone response we compared plasma corticosterone concentrations after acute shaker stress in mice that were exposed to daily shaker stress for 9 days. OT+/+ and OT−/− mice were divided into four groups. Conditioned stressed mice (OT+/+ n = 10, OT−/− n = 15) received 10 min of shaker stress daily between 0800 and 1200 for 9 days. On the 10th day of the experiment, animals were killed 10 min after shaker stress. Naive stressed mice (OT+/+ n = 11, OT−/− n = 12) were handled for 9 days and received acute stress on the 10th day of the experiment. Conditioned control mice (OT+/+ n = 5, OT−/− n = 7) received 10 min of shaker stress daily between 0800 and 1200 for 9 days. On the 10th day of the experiment, animals were killed without being exposed to shaker stress. Naive control mice (OT+/+ n = 9, OT−/− n = 8) were handled daily for 9 days, but not exposed to shaker stress, and were killed on the 10th day of the experiment. Trunk blood was collected at death to determine plasma corticosterone levels.

Experiment 4. Shaker stress induced Fos expression in the mouse brain. To evaluate the role of endogenous OT in the activation of hypothalamic and forebrain areas associated with the stress response to psychogenic stress, mice of both genotypes were exposed to shaker stress. Female OT+/+ and OT−/− mice were exposed to
10-min shaker stress (n = 3 of each genotype) or control conditions (n = 3 of each genotype) and returned to their home cages. Sixty to seventy-five minutes later, mice were anesthetized by intraperitoneal injection of ketamine-xylazine. Mice were perfused transcardially with 0.15 M saline followed by 4% paraformaldehyde fixative (0.1 M sodium phosphate buffer containing 4% paraformaldehyde, 1.4% t-lysine, and 0.2% sodium metaperiodate). Fixed brains were removed from the skull, postfixed in 4% paraformaldehyde at 4°C for 12–18 h, and transferred to 25% sucrose solution (4°C) for 24–72 h before sectioning. Coronal tissue sections were cut (35 μm thick) using a freezing stage microtome and collected in four serially adjacent sets. Tissue was stored in cryopreservant solution (66) at −20°C until immunocytochemical processing.

Sections were rinsed in several changes of 0.1 M sodium phosphate buffer, treated for 30 min in 1% sodium borohydride (Sigma, St. Louis, MO), and rinsed again in buffer. Antisera were diluted in buffer containing 0.3% Triton X-100 and 1% normal donkey serum. Tissue sections were incubated for 48 h at 4°C in rabbit anti-Fos (1:50,000; provided by Dr. Philip Larsen, Rheoscience, Denmark), rinsed, and then incubated in biotinylated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature. Sections were rinsed and processed using the Vectastain Elite avidin-biotin immunoperoxidase method (Vector Laboratories, Burlingon, CA). A solution of DAB, nickel sulfate, and H2O2 was used to generate blue-black nuclear c-Fos immunolabeling. The tissue was then processed for immunoperoxidase localization of OT (rabbit anti-OT; 1:30,000; Chemicon, Temecula, CA), AVP (rabbit anti-AVP; 1:20,000; Chemicon), or CRH (rabbit anti-CRH, 1:10,000; Peninsula Laboratories, Belmont, CA) using a nonenhanced DAB reaction to create a brown cytoplasmic immunoperoxidate. Immunolabeled tissue sections were mounted onto Super Frost Plus glass slides (Fisher Scientific, Pittsburgh, PA), cleared in graded alcohols and xylene, and placed under a coverslip using Histomount (VWR, Bridgeport, NJ).

A quantitative analysis of Fos expression in OT-, AVP-, and CRH-positive neurons within the PVN and supraoptic nucleus (SON) was conducted in anatomically matched tissue sections. For this purpose, anatomically matched brain sections containing OT-positive neurons (in OT+/+ mice only, because OT immunolabeling is absent in OT−/− mice; 4–5 brain sections from bregma −0.58 to −1.22 mm), AVP-positive neurons (in all mice; 4–5 brain sections from bregma −0.58 to −1.22 mm), and CRH-positive neurons (in all mice; 2–3 brain sections from bregma −0.58 to −0.94 mm) were selected for analysis (24). The PVN was divided into anterior and posterior sections using anatomic landmarks previously described in mouse (49). Unlike in rats, in which parvocellular and magnocellular divisions of the PVN are easily distinguished, there is as yet no conventional method to separate these two divisions in mice. OT, AVP, and CRH cells were considered Fos-positive when their nuclei contained blue-black immunoreactivity. The average numbers of activated, phenotypically identified neurons within the PVN and SON per section (both sides) were calculated for each mouse.

A quantitative analysis of shaker stress-induced Fos expression was also conducted in the BNST (combined counts of the dorsal lateral, ventral lateral, anterior medial, ventral medial nuclei), MeA, CeA, mPOMA, and the paraventricular thalamic nucleus (PVA) of OT+/+ and OT−/− mice. In each mouse, brain sections containing anatomically matched levels of the BNST (3–4 brain sections in bregma 0.62 to 0.26 mm), the MeA (4–6 brain sections in bregma −0.82 to −1.70 mm), the CeA (6–8 brain sections in bregma −0.82 to 1.70 mm), the mPOMA (3–5 brain sections in bregma 0.5 to −0.10 mm), and the PVA (3–5 brain sections in bregma −0.22 to −0.82 mm) were selected for analysis (24). Cells were considered Fos positive when their nuclei contained blue-black immunoreactivity, regardless of intensity. The average number of cells per section (both sides) in each brain region was calculated for each mouse.

Hormone Analyses

Trunk blood was collected into heparinized tubes on ice, centrifuged at 4°C, and plasma was stored at −20°C until assay. Plasma corticosterone levels were measured by radioimmunoassay using a commercial kit purchased from Diagnostic Products (Los Angeles, CA). The detection limit for corticosterone is 5 ng/ml.

Statistics

Results are expressed as group means ± SE. Multiple group comparisons for differences in corticosterone levels were analyzed by repeated-measures ANOVA. The number of Fos-immunoreactive AVP and CRH neurons of the PVN and Fos-positive neurons in limbic brain areas of control and shaker stress-exposed OT+/+ and OT−/− mice was also analyzed using repeated-measures ANOVA. When the overall F ratio was significant, pairwise comparisons were made with the Bonferroni/Dunn post hoc comparison. Pairwise treatment-related Fos-positive OT neurons in the PVN were analyzed by a two-tailed t-test. Significance was set at P < 0.05.

RESULTS

Experiment 1A. Corticosterone Response to Shaker Stress in Group-Housed Mice

In group-housed mice, plasma corticosterone concentrations increased significantly in mice of both genotypes at 10 and 30 min posttermination of a 10-min shaker stress (ANOVA, F1,25 = 26.60, P < 0.0001; Fig. 1A) compared with corticosterone levels in nonstressed, control mice. The increase in plasma corticosterone was similar 10 and 30 min after shaker stress. Furthermore, OT−/− mice released more corticosterone than OT+/+ mice 10 min (P < 0.0002) and 30 min (P < 0.04) posttermination of shaker stress.

Experiment 1B. Corticosterone Response to Shaker Stress in Individually Housed Mice

The study was repeated in individually housed mice. As was the case with group-housed mice, plasma corticosterone concentrations increased in both genotypes 10 min after termination of a 10-min shaker stress compared with mice not receiving this stress (ANOVA, F1,26 = 8.56, P < 0.007). Moreover, corticosterone concentrations increased to a greater degree in OT−/− mice vs. OT+/+ mice (ANOVA, F1,12 = 51.15, P < 0.02; Fig. 1B).

Experiment 2. Corticosterone Response to Shaker Stress During the Estrous Cycle

OT−/− mice released more corticosterone than OT+/+ mice when exposed to shaker stress (ANOVA, F1,30 = 32.08, P < 0.0001; Fig. 2). Significant genotypic differences in the corticosterone response to shaker stress were observed at each stage of the estrous cycle. Corticosterone increase in response to shaker stress was higher in OT−/− compared with OT+/+ mice in the diestrous (P < 0.03), proestrous (P < 0.004), and estrus (P < 0.003) stages of the estrous cycle (Fig. 2).

Plasma corticosterone levels of all mice (OT+/+ and OT−/− mice) after shaker stress fluctuated across the estrous cycle (ANOVA, F2,26 = 9.03, P < 0.008). Corticosterone levels in response to shaker stress were lower in OT+/+ and OT−/− mice during estrus than during diestrus (P < 0.003) or proestrus (P < 0.003). Because there was not an interaction between genotype and stage of the estrous cycle (ANOVA,
conditioned stressed OT of both genotypes (Fig. 3). The corticosterone response of conditioned control mice were the same as naive control mice episode of acute shaker stress. Basal corticosterone levels of shaker stress was compared with mice receiving a single Shaker Stress Experiment 3. Corticosterone Response to Repeated verified 4-day estrous cycles in both genotypes. Vaginal cytology rone response to shaker stress across the estrous cycle in each individual genotype could not be determined. Vaginal cytology sterile response to shaker stress in both genotypes. Corticosterone concentrations were also measured in individually housed OT+/+ and OT−/− mice 10 min after termination of a 10-min shaker stress or in control mice that did not receive shaker stress. Plasmatic corticosterone concentrations increased in both genotypes after shaker stress, but to a greater degree in OT−/− mice 10 min after termination of a 10-min shaker stress or in control mice that did not receive shaker stress. Plasma corticosterone concentrations increased in both genotypes after shaker stress, but to a greater degree in OT−/− vs. OT+/+ mice at 10 min. The number of mice per group is located in or above the data bars.

F2,30 = 0.881, P > 0.05), pairwise contrasts of the corticoste rone response to shaker stress across the estrous cycle in each individual genotype could not be determined. Vaginal cytology verified 4-day estrous cycles in both genotypes.

Experiment 3. Corticosterone Response to Repeated Shaker Stress

The corticosterone response of mice repeatedly exposed to shaker stress was compared with mice receiving a single episode of acute shaker stress. Basal corticosterone levels of conditioned control mice were the same as naive control mice of both genotypes (Fig. 3). The corticosterone response of conditioned stressed OT+/+ (P < 0.04) and OT−/− (P < 0.01) mice was attenuated compared with naive stressed mice (Fig. 3). However, repeated exposure to shaker stress did not abolish the difference in corticosterone release between genotype. Conditioned stressed OT−/− mice had higher plasma corticosterone concentrations compared with OT+/+ mice (P < 0.01, Fig. 3), despite repeated exposure to shaker stress.

Experiment 4. Shaker Stress Induced Fos Expression in the Mouse Brain

As expected, similar numbers of OT-immunoreactive neurons were counted in the PVN of control OT+/+ mice (99 ±
3 OT cells per section) and in OT+/+ mice exposed to shaker stress (92 ± 12 OT cells per section; P > 0.05; Table 1). OT-immunoreactive neurons were not observed in OT−/− mice (Fig. 4, A and B). Ten minutes of shaker stress robustly activated Fos in the PVN of OT+/+ and OT−/− mice compared with Fos activation in control mice. Shaker stress activated Fos in a small but significant subset of OT-positive magno- and/or parvocellular PVN neurons in OT+/+ mice (P < 0.007; Fig. 4, E and F, Table 1). Fos activation did not increase in the SON in either OT+/+ or OT−/− mice after shaker stress (Figs. 4, G and H, and 5, G and H).

Similar numbers of AVP-immunoreactive PVN neurons were counted in mice of both genotypes regardless of stress exposure condition (OT+/+, 111.6 ± 3.2 AVP cells per section in control mice and 97.27 ± 1.7 AVP cells per section in stressed mice; OT−/−, 89.6 ± 9.1 AVP cells per section in control mice and 97.0 ± 18.0 AVP cells per section in stressed mice; ANOVA, F1.8 = 1.186, P > 0.05; Table 1). In contrast to stressor-induced activation of OT neurons in OT+/+ mice, AVP-positive PVN neurons were not activated after shaker stress in mice of either genotype (Fig. 5, A-F).

No genotypic or treatment-related differences were present in the number of CRH-immunoreactive cells counted in the PVN (OT+/+, 116 ± 1 CRH cells per section in control mice and 124 ± 7.6 CRH cells per section in stressed mice; OT−/−, 108 ± 2 CRH cells per section in control mice and 126 ± 13 CRH cells per section in stressed mice; ANOVA, F1.6 = 3.884, P > 0.05; Fig. 6E, Table 1). Significantly greater numbers of CRH-positive PVN neurons were activated to express Fos in OT+/+ (ANOVA, F1.3 = 62.25, P < 0.004) and OT−/− mice (ANOVA, F1.3 = 44.81, P < 0.001) after shaker stress compared with activation in control mice (Fig. 6; Table 1). The number of CRH neurons expressing Fos after shaker stress was not different in OT−/− mice compared with OT+/+ mice (ANOVA, F1.8 = 6.72, P = 0.06).

Quantitative analysis of Fos labeling revealed similar baseline Fos immunoreactivity within the BNST, MeA, CeA, mPOA, and PVA in control mice of both genotypes (Fig. 7). Shaker stress in both genotypes significantly increased the number of Fos-positive neurons in each forebrain region. A modest but statistically significant effect of genotype on stress-induced Fos expression was observed in the MeA but not in the other regions (Fig. 7). Stress-induced activation of MeA neurons was lower in OT−/− mice compared with activation in OT+/+ mice after shaker stress (ANOVA, F1.8 = 9.379, P < 0.02; Fig. 7).

**DISCUSSION**

This study demonstrates that a congenital absence of OT is associated with higher plasma corticosterone concentrations and decreased Fos activation in the MeA of female mice exposed to a psychogenic stress. We hypothesized that OT−/− mice would manifest augmented activation of the HPA axis in response to stress. To test this hypothesis we exposed mice to platform shaker and measured plasma corticosterone concentrations as an index of HPA axis activation. The pattern of neuronal activation after shaker stress was determined by mapping Fos immunoreactivity in select brain regions. Mice of both genotypes that were exposed to platform shaker had higher plasma corticosterone concentrations and greater Fos immunoreactivity in CRH-positive neurons compared with control mice that were not exposed to shaker stress. Importantly, stress-induced plasma corticosterone concentrations were higher in female OT−/− than OT+/+ mice. Genotypic differences in the stress-induced release of corticosterone were present during each stage of the estrous cycle and persisted when mice were conditioned to daily shaker stress exposure. Diminished activation of medial amygdala neurons in OT−/− vs. OT+/+ mice suggests a potential neural correlate for the genotypic difference observed in the corticosterone response to shaker stress.

Shaker stress has been reported to release corticosterone and OT, but not AVP, within the PVN and plasma of rats (27, 45). We now report that shaker stress also promotes corticosterone release in mice and activates the majority of CRH neurons in the PVN. Shaker stress also activated OT neurons in the PVN of OT+/+ mice, but did not activate AVP neurons in either genotype and did not activate either OT or AVP neurons in the SON. AVP acts synergistically with CRH to stimulate anterior pituitary release of ACTH and, in turn, to facilitate corticosterone secretion (5, 53). The observed absence of AVP neural activation after shaker stress in both genotypes suggests that AVP is unlikely to account for the greater stress-induced release of corticosterone observed in OT−/− mice.

Unlike the situation in rats, in mice there appears to be no clear segregation of magnocellular and parvocellular OT or AVP neurons in the PVN. We administered Fluorogold retrograde tracer systemically and indeed confirmed that parvo- and mag-

---

**Table 1. Number of cells activated per section in the paraventricular nucleus of the hypothalamus stained for Fos activation and peptide immunoreactivity in control and shaker stress-exposed mice**

<table>
<thead>
<tr>
<th></th>
<th>OT+/+</th>
<th>OT−/−</th>
<th>OT+/+</th>
<th>OT−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>OT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number of neurons</td>
<td>111.6 ± 3.2</td>
<td>97.0 ± 18.0</td>
<td>126.97 ± 12.99</td>
<td></td>
</tr>
<tr>
<td>Percentage of neurons activated</td>
<td>0.34 ± 0.08</td>
<td>0.67 ± 0.07</td>
<td>0.73 ± 0.41</td>
<td></td>
</tr>
<tr>
<td><strong>AVP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number of neurons</td>
<td>111.57 ± 3.19</td>
<td>89.6 ± 9.07</td>
<td>124.17 ± 7.62</td>
<td></td>
</tr>
<tr>
<td>Percentage of neurons activated</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.67 ± 0.67</td>
<td></td>
</tr>
<tr>
<td><strong>CRH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number of neurons</td>
<td>116.0 ± 1.0</td>
<td>108.96 ± 2.29</td>
<td>126.97 ± 12.99</td>
<td></td>
</tr>
<tr>
<td>Percentage of neurons activated</td>
<td>1.83 ± 0.17</td>
<td>1.42 ± 0.08</td>
<td>94.4 ± 10.69</td>
<td></td>
</tr>
</tbody>
</table>

OT, oxytocin. Values are means ± SE. *P < 0.05 compared with control mice of the same genotype.
Fig. 4. Shaker stress-induced Fos activation in oxytocin neurons of the hypothalamus. Color photomicrographs illustrating Fos immunostaining (blue-black nuclei) in the paraventricular nucleus (PVN) of control OT−/− (A) and OT+/+ (C) mice and OT−/+ (B) and OT+/+ (D) mice exposed to shaker stress. Tissue sections are double labeled for Fos and OT (brown cells). E: arrows indicate cells that are double labeled for Fos and OT. F: bar graph depicting the percent of Fos-positive OT neurons within the PVN. Exposure to shaker significantly increased Fos expression in OT-positive neurons of the PVN. However, exposure to shaker stress did not result in increased Fos immunostaining in the supraoptic nucleus (SON) of OT−/− (G) or OT+/+ mice (H). III, third ventricle; otx, optic tract.

Fig. 5. Shaker stress-induced Fos activation in vasopressin neurons of the hypothalamus. Color photomicrographs illustrating Fos immunostaining (blue-black nuclei) in the PVN of control OT−/− (A) and OT+/+ (C) mice and OT−/+ (B) and OT+/+ (D) mice exposed to shaker stress. Tissue sections are double labeled for Fos and AVP (brown cells). Exposure to shaker did not increase Fos expression in AVP-positive neurons of the PVN of OT−/− (E) or OT+/+ mice (F). In addition, exposure to shaker stress did not result in increased Fos immunostaining in the SON of OT−/− (G) or OT+/+ mice (H).
narcocellar OT and AVP neurons are intermingled within the PVN of OT−/− and OT+/+ mice (unpublished observations). Fluorogold labeling in this procedure identifies brain and spinal cord neurons whose axon terminals lie outside the blood-brain barrier, including hypothalamic endocrine neurons (35, 41). It is not yet clear whether shaker stress activates parvocellular, magnocellular, or both subtypes of OT PVN neurons in the mouse. However, future studies combining immunohistochemical labeling of Fos and OT in mice after Fluorogold injections will help to answer this question. In the rat, AVP neurons that coexpress CRH and respond to stress are located in the medial parvocellular subdivision of the PVN (61). Basal expression of AVP in these neurons is below the limits of immunocytochemical detection unless stimulated by either adrenalectomy (55, 56) or enhanced by administration of colchicine (55, 56). Hence without prior treatment, standard immunohistochemistry may not readily identify these CRH/AVP neurons. We have not yet performed dual labeling of CRH and AVP to determine if mice, like rats, also have a subpopulation of AVP neurons that coexpress CRH. Although Fos activation was not observed in the AVP-positive PVN neurons in either genotype after shaker stress, we cannot be certain that the level of AVP peptide was sufficiently abundant in all AVP neurons to be detected by immunohistochemistry. Additional techniques such as measurement of AVP ribonucleic acid (both heteronuclear and messenger) in the PVN of stressed mice or AVP peptide in the external zone of the median eminence of stressed mice may help address these possibilities. Although dual-labeling immunohistochemistry identified selective activation of Fos in OT, but not AVP, PVN neurons after shaker stress in mice, we cannot exclude the possibility that different analytic techniques may identify differences in AVP expression between genotypes.

Exposure to shaker stress resulted in an increased corticosterone response in both group and individually housed female OT−/− mice compared with OT+/+ mice. Housing conditions (i.e., group vs. individual) have been shown to influence the stress and anxiety response in rodents. Group-housed rats show lower levels of corticosterone (9) and decreased anxiety-related behavior compared with individually housed rats (48). Furthermore, OT is a neuropeptide associated with social behavior (74). Chronic administration of OT into the brains of male rats increased the amount of social interaction with other male rats (71). To ensure that the genotypic differences in plasma corticosterone were due to shaker stress exposure and not due to social environment, female OT+/+ and OT−/− mice were both group and individually housed before testing. We conclude that the enhanced corticosterone release evaluated in female OT−/− mice compared with OT+/+ mice was not due to housing conditions or social environment.

The peak and nadir circadian concentrations of corticosterone occur during dark and light hours, respectively, in mice of both genotypes and the magnitude of the circadian peak does not differ between genotypes (2). Because greater variability in basal corticosterone concentrations can be expected at the circadian peak of corticosterone secretion, we conducted our studies in the early morning, at the circadian nadir of corticosterone secretion when fluctuations in plasma corticosterone secretion are at a minimum. Therefore, it is unlikely that higher plasma concentrations of corticosterone in stressed OT−/− compared with OT+/+ mice are due to spontaneous plasma corticosterone fluctuation. Indeed, plasma corticosterone concentrations in nonstressed mice did not differ between genotypes. Additionally, by obtaining comparison blood samples in both genotypes at the same time of day, differences in corticosterone due to circadian variation were minimized.

Importantly, the genotypic difference in the corticosterone response to shaker stress was independent of stage of the estrus cycle. OT−/− mice exposed to shaker stress released more corticosterone than OT+/+ mice during each stage of the estrous cycle. Furthermore, estrous cyclicity (as determined by...
vaginal smears) was similar in both genotypes. Thus the stress-induced hyperresponsiveness of OT+/H11002/H11002 vs. OT+/H11001/H11001 mice was present regardless of the stage of the cycle. Both basal (7, 12) and stress-induced release of plasma corticosterone (65) have been reported to vary across the estrous cycle of rats and to be highest at proestrus, the time of maximal estrogen secretion (25). A Medline search of the scientific literature from 1970 to 2004 yielded only one report of stress-induced corticosterone concentrations across the estrous cycle of BALB/c mice (59), but no reports exist regarding the C57BL/6 background mouse strain used in the present study. The primary intent of this study was not to determine if the magnitude of the stress-induced release of corticosterone differed across the estrous cycle of mice. Nevertheless, we did identify lower stress-induced corticosterone concentrations after shaker stress during estrus compared with diestrus or proestrus, and this difference was observed in OT+/H11001/H11001 and OT+/H11002/H11002 mice.

Several reports about the adaptation of the HPA axis to repeated stress in rats have been published (28, 32, 33, 36). Repeated exposure of rats to the same stressor results in the habituation of the corticosterone response to that stressor. The corticosterone response to shaker stress was attenuated if rats were repeatedly exposed to this stress (27). Similarly, in the present study, mice of both genotypes that were habituated to daily shaker stress for 9 days had a lower plasma corticosterone response to shaker stress on the 10th day compared with responses of naive mice subject to the same stress only once. Despite the attenuated corticosterone response observed in mice of both genotypes, OT−/− mice still had a significantly higher plasma corticosterone response to shaker stress compared with OT+/+ mice. These findings support the view that endogenous OT attenuates the corticosterone response to repeated or acute shaker stress. However, OT by itself cannot account for the attenuation of corticosterone to repeated stress exposure, because habituation was also observed in OT−/− mice.

Our findings in mice are consistent with evidence that central OT attenuates stress-induced activation of the HPA axis in rats (69, 70). CRH stimulates the anterior lobe of the pituitary gland to release ACTH, which in turn stimulates the synthesis and release of corticosterone from the adrenal gland (4, 67). Both genotypes display equivalent corticosterone responses to exogenous CRH administered intracerebroventricularly (2). Hence, OT−/− mice display the same ability as wild-type mice to release ACTH and in turn corticosterone after central CRH administration. Thus genotypic differences in pituitary-adrenal responsiveness to CRH seem an unlikely explanation for the higher stress-induced corticosterone concentrations in OT−/− mice.

To determine whether the lack of OT may alter stress-induced activation of CRH neurons in the PVN, we performed double immunohistochemistry for Fos and CRH in hypothalamic brain sections of control and shaker stress-exposed mice.

Fig. 7. Shaker stress-induced neural activation of the limbic forebrain. Shaker stress significantly activated Fos in the bed nucleus of the stria terminalis (BNST; ANOVA, F1,8 = 54.75, P < 0.0001), medial (MeA; ANOVA, F1,8 = 159.97, P < 0.0001) and central (CeA; ANOVA, F1,8 = 9.11, P < 0.02) nuclei of the amygdala, medial preoptic area (mPOA; ANOVA, F1,8 = 39.74, P < 0.0002), and the paraventricular thalamic nucleus (PVA; ANOVA, F1,8 = 69.99, P < 0.0001). Shaker stress significantly activated Fos in all the brain areas evaluated compared with control mice of both genotypes. The number of Fos-immunoreactive cells was lower in the MeA of OT−/− mice than OT+/+ mice exposed to shaker stress.
of both genotypes. Shaker stress activated Fos in CRH-positive neurons in the PVN of both genotypes, and the number of activated CRH neurons was significantly greater than in control mice. The number of activated CRH-positive neurons tended to be higher in OT−/− mice than in OT+/+ mice, although this difference did not reach statistical significance (P = 0.06). Recently, male OT−/− mice exposed to 4 h of restraint stress were reported to have greater abundance of CRH mRNA compared with OT+/+ mice (46). Unlike in situ hybridization histochemistry that measures CRH mRNA expression, double immunohistochemistry for Fos and CRH is an assessment of the activation of CRH-positive neurons. Therefore, our findings are a quantitative assessment of the population of CRH neurons activated in response to shaker stress. These findings may not be reflective of the abundance of CRH mRNA expressed in response to shaker stress. Future experiments may reveal a statistically significant genotype-related increase in CRH activation if mice are exposed to a longer or more intense period of platform shaker, because lack of OT appears to result in increased CRH activation and expression (46) in mice exposed to psychogenic stressors.

There is a growing body of evidence that OT−/− mice display enhanced stress and anxiety responses relative to wild-type mice. Female OT−/− mice display increased anxiety-related behavior in the EPM (38) and increased stress-induced hyperthermia in response to a novel environment (2) compared with OT+/+ mice. Limbic forebrain pathways are activated by psychogenic stressors requiring higher-order processing of a stimulus, such as shaker stress in rats (27, 45), restraint stress in male OT−/− mice (46), or restraint stress in female rats (69). OT signaling pathways in the limbic forebrain are anatomically positioned to modulate the HPA axis response to stress. In the present study, exposure to shaker stress led to increased Fos expression in the limbic forebrain of OT−/− and OT+/+ mice. Moreover, OT−/− mice displayed significantly less Fos activation in the MeA after shaker stress compared with MeA activation in OT+/+ mice. The MeA in particular appears to be a target area for the actions of OT. Decreased Fos activation in the MeA of OT−/− vs. OT+/+ mice also has been reported in male mice after restraint stress (46) and in a social memory task (22), suggesting differences in forebrain processing of stimuli related to these stressors. Moreover, infusion of synthetic OT into the lateral ventricle of ovariectomized rats exposed to restraint stress reduced stress-induced Fos activation in specific forebrain regions (69). These data collectively suggest that the MeA is not only involved in behavioral responses such as social behavior and anxiety but that it also may play an important role in HPA axis responsiveness to stress. No studies to date have reported OT release within the MeA of mice during stress, although the present results indicate that a genetic absence of OT is associated within diminished stress-induced activation of the MeA.

The PVN receives little direct input from the medial amygdala, but the MeA sends more robust inhibitory GABAAergic projections to the BNST and mPOA in rats (10), which in turn send GABAAergic projections to the medial paraventricular PVN (51). Consequently, activation of the MeA results in activation of the HPA axis via disinhibition of inhibitory limbic forebrain projections to the PVN. Similar studies have not been performed in the mouse. However, if the projections from the MeA of the mouse are similar to those reported in the rat, then the MeA may emerge as a principal brain area that can account, at least in part, for heightened stress-induced corticosterone response in OT−/− mice.

ACKNOWLEDGMENTS
The authors acknowledge E. A. Myers for the assistance with the immunohistochemistry and Dr. Hou ming Cai for assistance with the animal studies.

GRANTS
The work was supported by National Institutes of Health Grant HD-37268 (to J. A. Amico).

REFERENCES
OXYTOCIN AND STRESS


