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Differential Expression of Vasopressin, Oxytocin and Corticotrophin-Releasing Hormone Messenger RNA in the Paraventricular Nucleus of the Prairie Vole Brain Following Stress

Y. Liu,* J. T. Curtis,* C. D. Fowler,* C. Spencer,† T. Houpt† and Z. X. Wang*
*Department of Psychology and †Department of Biology, Neuroscience Program, Florida State University, Tallahassee, FL 32306, USA.

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Abstract

Forced swimming, as an effective stressor, has been found to facilitate the development of pair bonds in male but to interfere with this behaviour in female prairie voles (*Microtus ochrogaster*). In the present study, we found that forced swimming differentially influenced the expression of messenger RNA for vasopressin, oxytocin and corticotrophin-releasing hormone (CRH) in the paraventricular nucleus of the hypothalamus (PVN) in the prairie vole brain. Forced swimming did not alter vasopressin mRNA labelling, but did induce a sustained decrease in oxytocin mRNA labelling and a progressive increase in CRH mRNA labelling in the PVN. The elevated CRH mRNA labelling appeared to be due to an increased number of cells synthesizing CRH mRNA and an enhanced ability of individual cells to produce CRH mRNA. Male and female prairie voles did not differ in the vasopressin, oxytocin or CRH mRNA expression either at the basal levels or in response to swimming stress. Together, these data indicate that the hypothalamic response of vasopressin, oxytocin and CRH messenger RNAs to swimming stress is regulated by distinct transcriptional factors. In addition, it seems unlikely that these changes are involved directly in the sex differences in pair bond formation.

Introduction

The paraventricular nucleus of the hypothalamus (PVN) plays an important role in the mediation of adaptive neuroendocrine responses to stress. In the PVN, the corticotrophin-releasing hormone (CRH) is synthesized. CRH acts as a releasing factor for adrenocorticotrophic hormone (ACTH) and therefore is the primary hypothalamic regulator activating the hypothalamic–pituitary–adrenocortical (HPA) axis (1). Neurones in the PVN also produce other hypothalamic hormones, including vasopressin and oxytocin. Both vasopressin and oxytocin are sent through the neurohypophysial tract into the posterior pituitary, where they are released into circulation (2, 3). Vasopressin and oxytocin genes share considerable homology and a common ancestral origin, and sometimes respond similarly to physiological stimuli (4). Some neurones in the PVN terminate in the external zone of the median eminence and secrete vasopressin and oxytocin into the pituitary portal circulation, where vasopressin and oxytocin act also as releasing factors for ACTH and regulate the HPA axis activities (1, 5). Vasopressin is generally considered to be a weak regulator and acts synergistically with CRH (6). Different patterns of vasopressin and/or CRH mRNA responses have been reported in the PVN depending upon the stressor and stress paradigm used (7–11). In the case of oxytocin, beside its evident importance during parturition and lactation, it is also activated in response to stressful stimuli although its involvement may be completely different from that of vasopressin (12).

The fact that stress induces hypothalamic neuroendocrine responses which, in turn, alter subsequent behaviour has been studied in several animal models, including the prairie vole (*Microtus ochrogaster*). The prairie vole is a highly social monogamous rodent (13). In the laboratory, male and female prairie voles form pair bonds after mating (14, 15). Previous studies have demonstrated that swimming stress exerts gender-specific effects on the behaviour of prairie voles; it facilitates the development of pair bonds in males

Correspondence to: Zuoxin Wang PhD, Department of Psychology, Florida State University, Tallahassee, FL 32306–1270, USA (e-mail: zwang@psy.fsu.edu).

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but interferes with pair bond formation in females (16). Interestingly, swimming stress induces a similar increase in the level of circulating corticosterone in both male and female prairie voles (17, 18), but corticosterone treatment influences pair bonds in a gender-specific manner, similar to swimming stress (16). Because earlier studies also implicate vasopressin and oxytocin in pair bond formation in prairie voles (19–21), it has been suggested that in males, corticosterone and vasopressin may interact to modulate pair bonding, whereas in females, acute stress or exposure to corticosterone may inhibit the release of oxytocin, impeding the development of pair bonding (16). In view of the stress effect on pair bond formation and the possible interaction of hypothalamic hormones, the present study was undertaken to examine the effects of forced swimming on the messenger RNA expression for vasopressin, oxytocin and CRH in the PVN of prairie voles. We hypothesized that swimming stress would affect vasopressin, oxytocin and/or CRH mRNA expression differentially in male and female prairie voles.

Materials and methods

Subjects

Subjects were sexually naive male and female prairie voles (*M. ochrogaster*) that were the F3 generation of a laboratory breeding colony started from field captured animals. Breeding pairs were housed in plastic cages (44 × 24 × 20 cm) that contained cedar chips as bedding. Water and food were provided *ad libitum*. All cages were maintained on a 14 : 10-h light : dark cycle with lights on at 0700. The temperature was maintained around 20 ± 1 °C. Voles were weaned at 3 weeks of age, housed in same-sex sibling pairs in plastic cages, and maintained under the same conditions as their parents.

Experimental procedures

At 60–70 days of age, subjects were randomly assigned to one of four treatment groups (n = 6 per sex in each group). In the first three groups, each subject was placed into a tank (44 × 24 × 20 cm) containing water of 10 cm depth at 32 °C for 5 min of forced swimming. The tank was emptied and cleaned between animals. This treatment, as a stressful stimulus, has successfully induced increases in glucorcoticoid concentrates in both male and female prairie voles (16, 17). Following forced swimming, voles were returned to their home cages and were then decapitated at 1 h, 3 h or 6 h later. Voles in the fourth group (control) were not subjected to forced swimming and remained in their home cages without disturbance. These voles were randomly decapitated at 1 h, 3 h or 6 h together with subjects from other groups. After decapitation, brains were removed and frozen on dry ice. Coronal sections (15 μm) through the paraventricular nucleus of the hypothalamus were cut on a cryostat and thaw-mounted onto Superfrost/Plus slides (Fisher). Slides were stored at −80 °C until processing for vasopressin, oxytocin or CRH *in situ* hybridization.

Probe preparation

Vasopressin and oxytocin oligoprobes were made according to the procedures described previously (22). The vasopressin probe consists of 48 bases and is complementary to the rat mRNA encoding amino acids 129–144 of the vasopressin precursor peptide. The oxytocin probe consists of 42 bases and is complementary to the rat mRNA encoding amino acid 82–96 of the oxytocin-associated neurophysin peptide. Both probes were labelled at the 3′-end with 75 pmol ^{35}S-dATP using terminal deoxynucleoxytocinidyl transferase (TdT) in the terminal transferase buffer containing 15 pmol oligo-DNA at 37 °C for 1.5 h. Thereafter, ammonium acetate and *Escherichia coli* tRNA were added. ^{35}S-labelled probes were purified by adding equal volume of phenol/chloroform, and then precipitated by adding 2.5 V 100% ethanol. The precipitate was dissolved in 100–200 μl TED.

The CRH antisense probe (Rat CRH 118–1093) was transcribed using T3 RNA polymerase from *Hin*dIII-digested pBluescript II SK$^+$-rCRH, and CRH sense probe was transcribed using T7 RNA polymerase from the same plasmid digested with SmaI. Transcription was performed at 37 °C for 1 h in the transcription buffer containing 10 mM DTT, 0.2 mM ATP, GTP and UTP, 5 mMCTP, 25 ng/ml template DNA, 70 mCi of ^{35}S-CTP, 2 U/ml RNase inhibitor and 1–2 U/ml RNA polymerase. After digestion by RNase-free DNase for 15 min at 37 °C, ammonium acetate and *E. coli* tRNA were added. Procedures for purification and precipitation were the same as described above for the vasopressin and oxytocin probes.

In situ *hybridization*

Vasopressin and oxytocin *in situ* hybridization were performed as described previously (22). Briefly, two sets of slide-mounted brain sections at 75 μm intervals were fixed in 4% paraformaldehyde in phosphate buffer solution (PBS, pH 7.4) at 4 °C for 5 min, washed in cold PBS and then digested with 5 μg/mL proteinase K in TE buffer (50 mM Tris-HCl and 5 mM EDTA, pH 8.0) for 15 min, followed by 0.1 M triethanolamine (pH 8.0) containing 0.25% acetic anhydride for 10 min. Slides were washed in 2 × SSC, dehydrated in ethanol, immersed in chloroform for 6 min, transferred quickly to 100% ethanol followed by 95% ethanol and then air-dried. Prehybridization was performed by applying each slide with 120 μl hybridization buffer containing 2.5 mg/ml tRNA at 37 °C for 2 h. The hybridization buffer contained 62.5% formamide, 12.5% dextran sulphate, 37.5 mM NaCl, 10 mM Tris-EDTA (pH 8.0), 1.25 Dehardt's solution, and 10 mM dithiothreitol. Slides were then washed with 2 × SSC, dehydrated and air-dried. Hybridization was performed by applying each slide with 120 μl of the hybridization buffer containing 0.5 mg/ml tRNA and 4 pmol/ml ^{35}S-labelled vasopressin-probe or oxytocin-probe. Slides were covered with parafilm and hybridized at 37 °C for 16 h. Thereafter, slides were washed in 1 × SSC for 4 × 15 min at 55 °C, followed by 1 × SSC for 2 × 30 min at room temperature. To control for specificity, separate brain sections were processed for vasopressin or oxytocin *in situ* hybridization using the oxytocin or vasopressin sense probe. In each case, specific labelling was not detected.

For the CRH *in situ* hybridization, pretreatment, prehybridization and hybridization were similar to that of vasopressin or oxytocin *in situ* hybridization except that about 5×10^6 cpm/ml ^{35}S-CRH riboprobe was added in the hybridization solution. Wash was performed in 50% formamide and 0.1% β-mercaptoethanol in 2 × SSC at 55 °C for 2 × 15 min, followed by digestion in 25 μg/ml RNaseA in RNase buffer (8 mM Tris-HCl, 400 mM NaCl, 0.8 mM EDTA, pH 8.0) at 37 °C for 30 min and blockage in 1 mM DTT in the same buffer at 37 °C for 30 min. Thereafter, slides were washed again in 50% formamide and 0.1% β-mercaptoethanol in 2 × SSC at 55 °C for 2 × 15 min, and then washed in 50% formamide and 1% β-mercaptoethanol in 0.1 × SSC for 2 × 30 min at the same temperature. Slides were then air-dried. For specificity controls, separate brain sections were processed for CRH *in situ* hybridization using the sense probe, which did not result in any specific labelling.

All slides were put on BioMax MR film (Kodak) along with [^{14}C] microscales (Amersham, Chicago, IL, USA) for 1–3 days to generate autoradiograms for visualization of the mRNA labelling. Thereafter, slides were dipped into NBT-2 emulsion (Kodak) and exposed for 1 (for vasopressin or oxytocin mRNA) or 5 (for CRH mRNA) weeks at 4 °C. After developing, slides were stained with 0.5% Cresyl violet, dehydrated, immersed in xylene and coverslipped.

Data quantification and analysis

The density of vasopressin, oxytocin or CRH mRNA labelling in the PVN was first measured from the autoradiograms using a computerized image program (NIH IMAGE 1.60), permitting the conversion of the optical density to dpm/tissue equivalents from the standard curve derived from coexposed microscales. Sections on the autoradiograms were visually inspected. Four sections that contained the highest densities for each mRNA labelling in the PVN were measured bilaterally from each subject to provide individual means for data analysis. Data were analysed by a two-way analysis of variance (ANOVA) with treatment and gender as independent variables. Significant effects were further evaluated by a Student–Newman–Keuls (SNK) post hoc test.

Variations in the number of mRNA-labelled cells and/or grain density per labelled cell may contribute to the alteration in the density of mRNA labelling (23). Therefore, to further examine treatment effects on the CRH mRNA labelling in the PVN, we also counted the number of CRH-mRNA labelled cells and the grain density per labelled cell in the PVN from the emulsion-coated slides. The number of CRH-mRNA labelled cells for each subject was counted bilaterally in three sections (the same sections that were used for quantification from the autoradiograms). In addition, 10 cells were chosen stereologically on the same area of the PVN on each section (30 cells per

subject), and the grain density per labelled cell was counted using an imaging program (NIH IMAGE 1.6). These data were analysed by a two-way ANOVA followed by a SNK test. We also attempted to count the number of cells and grain density per labelled cell for the oxytocin and vasopressin mRNA labelling. However, the emulsion-coated slides were over-exposed, and overlapped grains prevented us from performing this analysis reliably.

Results

As reported in a previous study (22), dense clusters of vasopressin mRNA or oxytocin mRNA labelled cells were detected clearly in the PVN of the prairie vole brain. *In situ* hybridization also revealed CRH mRNA labelled cells in the PVN. Five min of forced swimming, as an acute stressor, differentially affected the levels of vasopressin, oxytocin and CRH mRNA in the PVN of the prairie vole brain. Overall, no gender differences were detected either at the basal levels or in response to swimming stress in any of the mRNA labelling measured in the PVN.

Forced swimming did not significantly alter vasopressin mRNA labelling in the PVN. In comparison to the control group, the levels of vasopressin mRNA labelling in the PVN varied slightly at 1, 3 or 6 h after forced swimming (Fig. 1 and Fig. 2A). On the other hand, the levels of oxytocin mRNA labelling in the PVN decreased significantly following forced swimming in both male and female prairie voles. Voles killed at 1, 3 or 6 h after forced swimming showed a significant decrease in the oxytocin mRNA labelling in the PVN relative to the control group ($F = 9.24$, $P < 0.001$; Fig. 1 and Fig. 2B). Among the three swimming-stressed groups sacrificed at different timepoints, no differences were found in either the vasopressin or oxytocin mRNA labelling.

Forced swimming elevated the level of CRH mRNA labelling in the PVN in both male and female prairie voles. Voles killed 6 h after forced swimming had a significant increase in the level of CRH mRNA labelling in the PVN ($F = 3.25$, $P < 0.05$) relative to the control females (Fig. 1 and Fig. 2C). In addition, voles killed at 1 h or 3 h after forced swimming also showed an increase in the CRH mRNA labelling in comparison to the control animals. These differences, however, did not reach statistical significance.

To analyse further the effects of swimming stress on the CRH mRNA labelling, we counted the number of CRH mRNA labelled cells and the grain density per labelled cell in the PVN from the emulsion-coated slides. Three hours after forced swimming, the subjects had a significant increase in the number of CRH-mRNA labelled cells in the PVN, and this increase was sustained through 6 h ($F = 3.57$, $P < 0.05$, Fig. 3 top panels and Fig. 4A). In contrast, the grain density per labelled cell increased 1 h after forced swimming and was sustained thereafter ($F = 4.70$, $P < 0.01$, Fig. 3 bottom panels

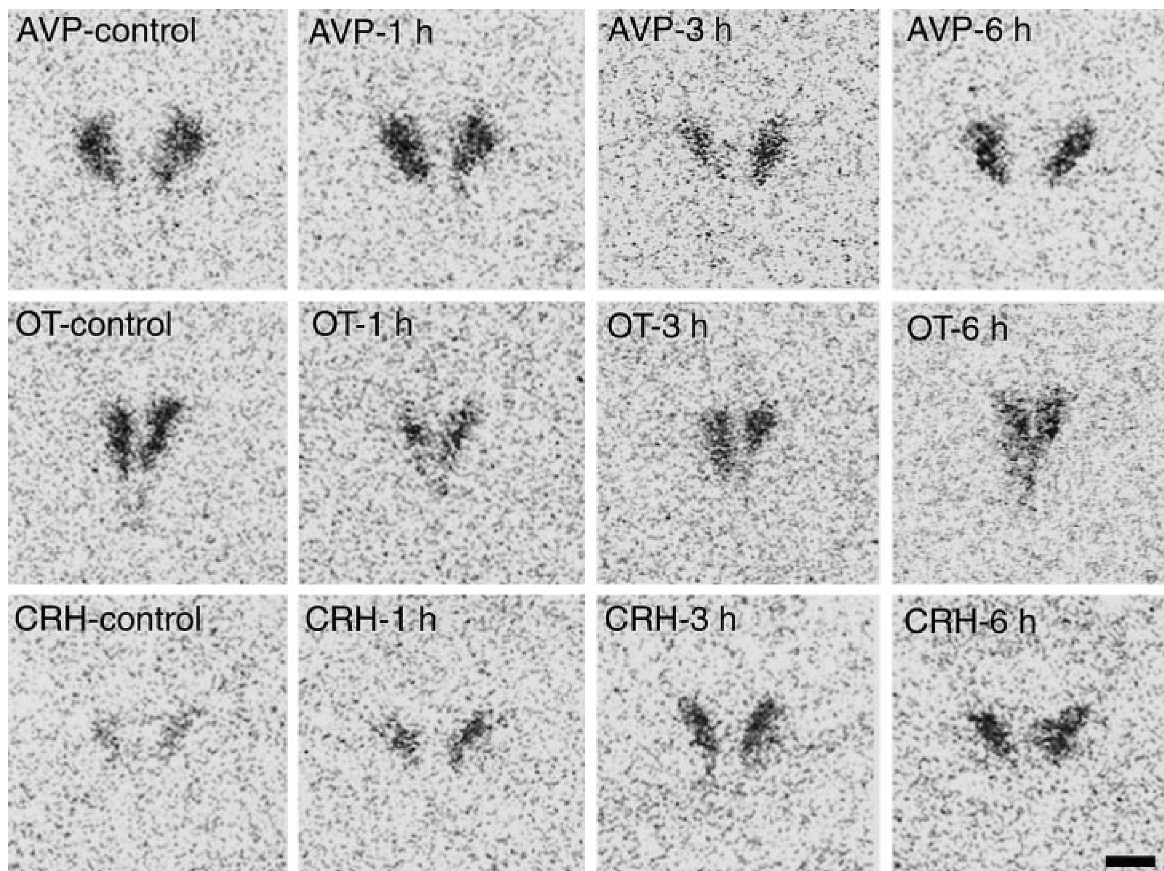

FIG. 1. Photomicrographs from the autoradiograms displaying the messenger RNA labelling for vasopressin, oxytocin or corticotrophin-releasing hormone (CRH) in the paraventricular nucleus of the hypothalamus in the prairie vole brain. Images were taken from control females (control) or females that were sacrificed at 1 h, 3 h or 6 h after forced swimming. Scale bar = 1 mm.

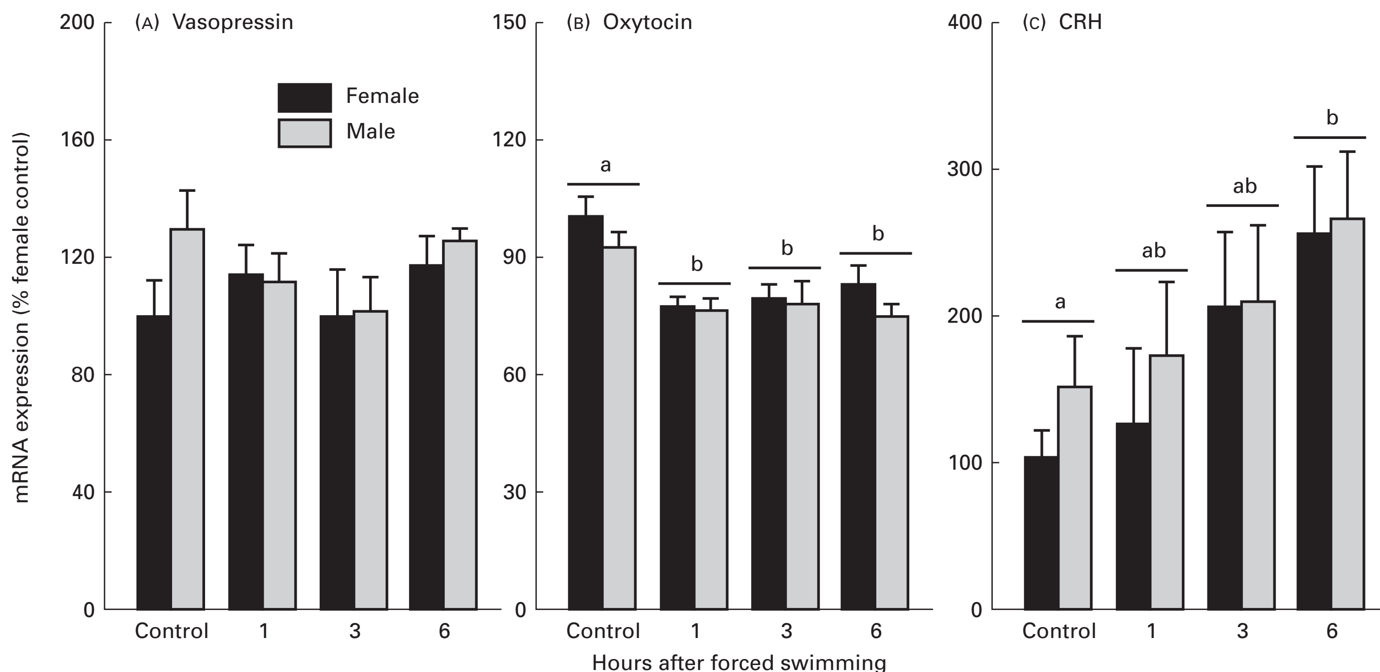

FIG. 2. The effects of forced swimming on the messenger RNA labelling for the hypothalamic hormones in the paraventricular nucleus of the hypothalamus in the prairie vole brain. Forced swimming did not significantly alter vasopressin mRNA labelling (A), but did induce a sustained decrease in oxytocin mRNA labelling (B) and a progressive increase in CRH mRNA labelling (C). Males and females did not differ in responses to swimming stress in any of the measured messenger RNAs. Data are presented as the percentages relative to control females. The alphabetic letters illustrate group differences following the SNK post hoc test. Groups (means ± SEM) labelled with different letters differ significantly from each other.

and Fig. 4B). No gender differences were detected in either the number of CRH-mRNA labelled cells or the grain density per labelled cell.

Discussion

Forced swimming is a non-escapable stressful situation (24) and as such is used widely for producing stress responses. In previous studies, forced swimming was found to enhance the levels of circulating corticosterone in both male and female prairie voles, and to facilitate the development of pair bonds in males but impair this behaviour in females (16–18). In the present study, we found that 5-min forced swimming had differential effects on the gene expression of the hypothalamic hormones; it did not alter vasopressin mRNA labelling, induced a sustained decrease in oxytocin mRNA labelling and triggered a progressive increase in CRH mRNA labelling in the PVN of the prairie vole brain. Furthermore, the increased CRH mRNA labelling appeared to be due to the increased number of CRH synthesizing cells and the enhanced ability of individual cells to synthesize CRH. Finally, no gender differences were found in the vasopressin, oxytocin or CRH mRNA labelling in the PVN either at the basal levels or in response to forced swimming, suggesting that the hypothalamic neuroendocrine response to swimming stress did not differ between male and female prairie voles at least at the timepoints examined.

After forced swimming, prairie voles showed a significant increase in CRH mRNA labelling in the PVN. These data are in agreement with the previous finding that swimming stress increases circulating corticosterone (17, 18), suggesting that forced swimming can serve as a mild stressor to activate the HPA axis in prairie voles. The demonstration that there was only a CRH, but not vasopressin or oxytocin, mRNA increase suggests that CRH is an important mediator of HPA axis activities of prairie voles in response to swimming stress. Furthermore, the increase in CRH mRNA was due to a combination of the increased number of CRH cells and enhanced ability or increased stability of individual cells to synthesize CRH (as indicated by increased grain density per cell). It is worth noting that the grain density increased significantly one hour after forced swimming whereas a significant increase in the number of CRH cells was not found until 2 h later. Such different time courses may imply different regulatory mechanisms for the transcription of CRH. It is possible that an initial response to swimming stress is to enhance the productivity of the existing CRH cells, and then the subsequent response includes recruiting more cells to produce CRH. In previous studies examining regulation of sex steroid hormones on vasopressin mRNA expression, changes in the number of vasopressin cells and grain density per cell both were attributed to the altered vasopressin mRNA labelling in the rat brain, indicating different regulatory mechanisms were involved (23, 25).

Transcription of both CRH and vasopressin mRNA could be activated by acute stress (26, 27). However, our data show that despite an increase in the CRH mRNA labelling, vasopressin mRNA labelling in the PVN of prairie voles did not change significantly in response to swimming stress. Under many experimental circumstances, parallel regulation of CRH and vasopressin has been observed

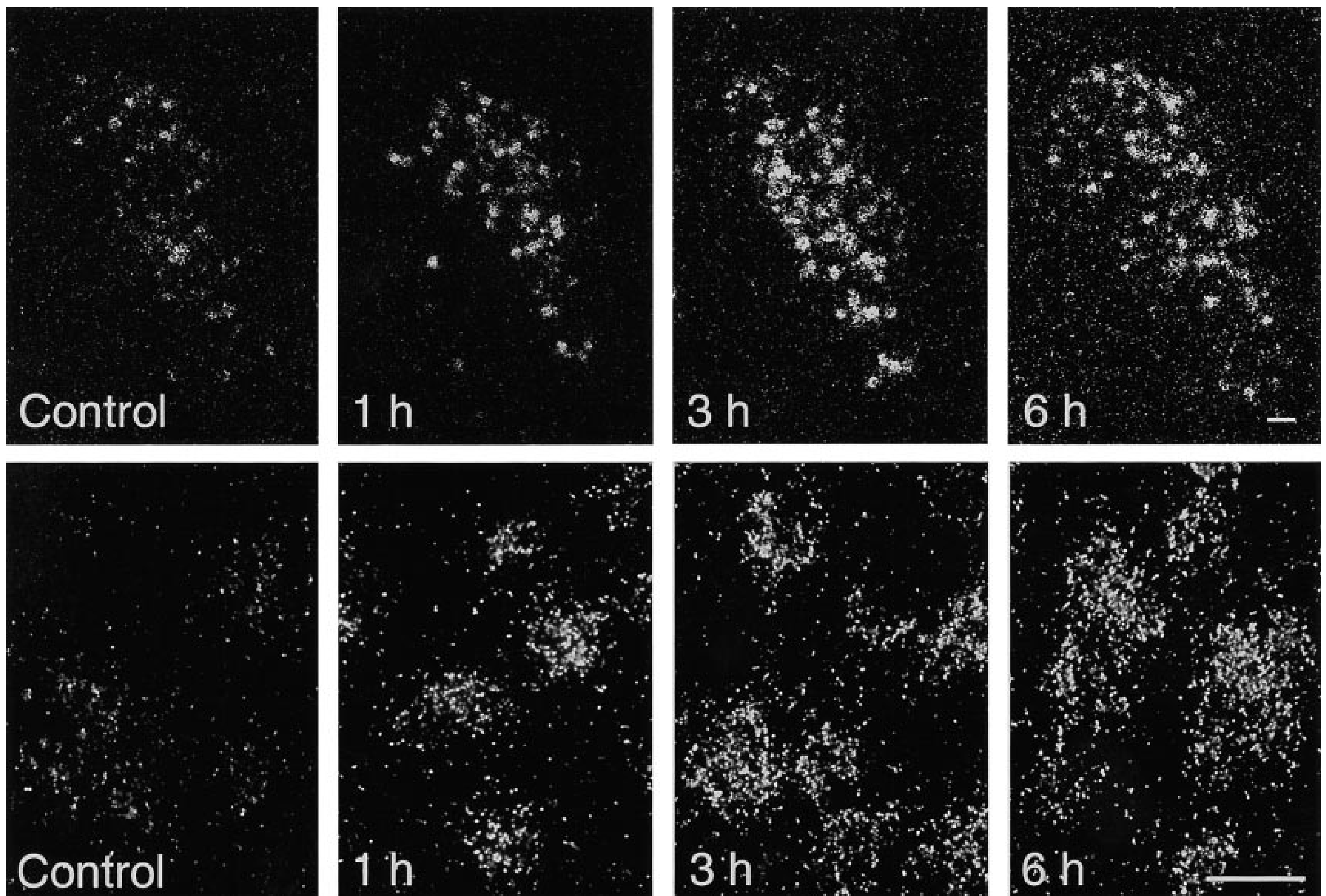

FIG. 3. Photomicrographs displaying CRH mRNA-labelled cells (top panels) and the grain density per cell (bottom panels) in the paraventricular nucleus of the hypothalamus in the prairie vole brain. Images were taken from the control females (control) or females that were sacrificed at 1 h, 3 h or 6 h after forced swimming. Scale bar = 20 μm.

(8, 26, 28, 29). It is recognized that, in rats, parvocellular neurones in the PVN consist of two populations: one in which CRH and vasopressin are coexpressed and another in which CRH is expressed alone (30). Therefore, a stress-induced concurrent increase in CRH and vasopressin mRNA can be interpreted by activation of CRH neurones that coproduce vasopressin (28, 29). As in the mouse (7, 31), it is difficult to distinguish anatomically between the parvocellular and magnocellular neurones in voles (22). In addition, it remains to be determined whether the vole's PVN contains different populations of neurones synthesizing CRH and vasopressin or CRH alone. Nevertheless, the disassociation in changes between CRH and vasopressin mRNA in response to swimming stress suggests that CRH and vasopressin are regulated by distinct transcriptional factors and/or are synthesized in different populations of the PVN neurones in the prairie vole brain. In recent studies in rats, acute stress was found to increase CRH mRNA expression in the PVN without altering the vasopressin mRNA labelling (10, 32).

The anatomical distribution and structural similarities of vasopressin and oxytocin might predict similar stress-induced vasopressin and oxytocin mRNA changes (4). However, the two have also been found to show independent responses under stress conditions (12). In our study, forced swimming induced a sustained decrease in oxytocin mRNA but had no effects on vasopressin mRNA labelling in the PVN. This difference in vasopressin and oxytocin mRNA changes after swimming stress provides further evidence indicating that different transcriptional mechanisms regulate gene expression in vasopressin and oxytocin neurones (12). It is interesting to note that decreased oxytocin mRNA labelling was associated with swimming stress and increased CRH mRNA labelling. Stress also down-regulates the hypothalamic oxytocin mRNA in the rat brain (33). In rats, CRH is colocalized with oxytocin in both parvocellular and magnocellular hypothalamic neurones (34–36) and oxytocin neurones also coexpress mRNA of CRH receptors (37). Therefore, CRH can modulate oxytocin mRNA by acting as autocrine or paracrine regulators (37). In addition, the increased level of corticosterone induced by swimming stress may negatively feedback to suppress oxytocin mRNA expression. Along the same line, the dissociation between vasopressin and CRH mRNA and the negative association between oxytocin and CRH mRNA in the vole brain provide further evidence supporting the notion that different neurone types have differential sensitivity and responsiveness to glucocorticoid feedback (26, 38).

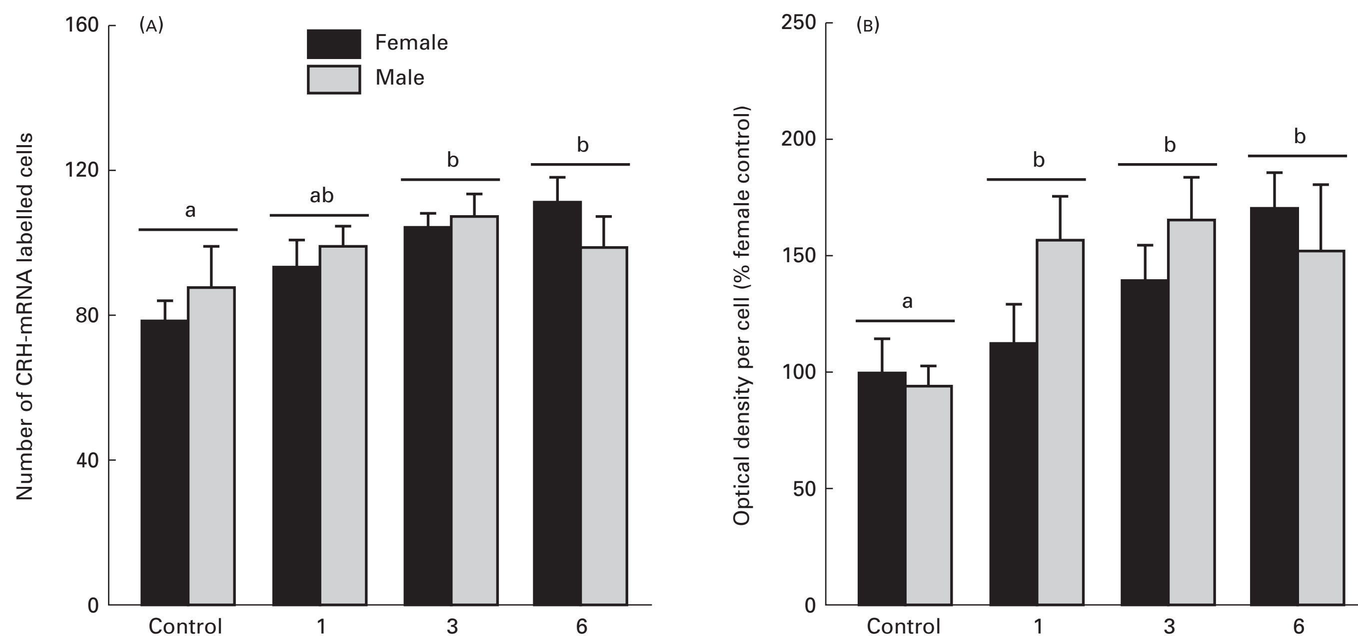

FIG. 4. The effects of forced swimming on CRH mRNA labelling in the paraventricular nucleus of the hypothalamus in the prairie vole brain. (A) At 3 or 6 h after forced SWIMMING, subjects had increased number of cells labelled for CRH mRNA, relative to control females. (B) Forced SWIMMING also elevated the grain density per CRH-labelled cell. Subjects sacrificed at 1, 3 or 6 h after forced swimming had an enhanced grain density per cell relative to control females. Male and female subjects did not differ either in the number of CRH-mRNA labelled cells or in the grain density per labelled cell in responses to swimming stress. The alphabetic letters illustrate group differences following the SNK posthoc test. Groups (means $\pm$ SEM) labelled with different letters differ significantly from each other.

Since forced swimming elevates the concentration of circulating corticosterone in both male and female prairie voles (17, 18), we predicted that swimming stress would increase CRH mRNA expression in the PVN similarly in both genders. This is confirmed in the present study. Furthermore, as forced swimming or corticosterone treatment influences pair bond formation differentially between male and female prairie voles (16), and vasopressin is implicated in the regulation of pair bonding in males (19) and oxytocin in females (20, 21), it has been hypothesized that swimming stress and the subsequent changes in CRH and corticosterone may interact with vasopressin or oxytocin in a gender-specific manner to regulate pair bonding (16). However, our data indicate that forced swimming did not influence the expression of vasopressin and oxytocin mRNA differentially between male and female prairie voles.

Several aspects of these results in regard to pair bond formation in voles are worth mentioning. First, oxytocin facilitates and oxytocin receptor blockade inhibits pair bond formation in female prairie voles (20, 21). Our data show that oxytocin mRNA labelling in the PVN decreases following forced swimming, which is in agreement with the notion that inhibition of oxytocin by swimming stress and elevated corticosterone impedes the development of pair bonding in female prairie voles (16). However, these data cannot explain why swimming stress facilitates pair bond formation in males (16). One possible explanation is that although forced swimming did not induce significant changes in the vasopressin mRNA expression, it might influence vasopressin release and/or vasopressin receptors and thus changes in vasopressin release or its actions on receptors may have been responsible for the altered behaviour in male prairie voles. An additional complication is that recent studies have also shown that oxytocin and vasopressin may interact to regulate pair bonding in male and female prairie voles, although the underlying cellular and physiological mechanisms are still unknown (39, 40). It is also possible that elevated levels of CRH and corticosterone induced by swimming stress may interact with other neurotransmitter or hormonal systems to regulate pair bonding [e.g. dopamine (41, 42)], and thus gender differences in those systems may account for the differences in behaviour. Finally, we examined only the messenger RNA expression at 1, 3 and 6 h after forced swimming. In prairie voles, a significant elevation in circulating corticosterone is detected 5 min after forced swimming (17). In rats, corticosterone alters vasopressin mRNA labelling in the PVN within 15 min of administration (26). Therefore, forced swimming may induce rapid changes in the synthesis and release of vasopressin and oxytocin, and the temporal patterns of those effects need to be examined in further studies.

In summary, we have demonstrated a complex coordinated response of gene expression for hypothalamic hormones in the prairie role brain to swimming stress. Forced swimming resulted in an increase in CRH mRNA, a decrease in OT mRNA, and no change in AVP mRNA labeling in the PVN. The increased number of cells and enhanced grain density per cell both contributed to the altered CRH mRNA labeling. In addition, male and female prairie voles responded to swimming stress with a similar hypothalamic neuroendocrine profile, despite their reported differences in behaviour. The functional significance, specifically the involvement and interaction of the

altered hypothalamic AVP, OT, and CRH in the regulation of pair bond formation, remains to be investigated.

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