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# Female responses to acute and repeated restraint stress differ from those in males ☆

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ABSTRACT

Chronic stress is implicated in diseases which differentially affect men and women. This study investigated how the activation of neuronal subpopulations contributes to changes in neuroendocrine regulation that predispose members of each sex to stress-related health challenges. Adult male and female rats were restrained in single (acute) or 14 consecutive daily (repeated) 30 min sessions; brain sections were immunohistochemically stained for Fos, arginine vasopressin (AVP) or glucocorticoid receptor (GR) within the paraventricular hypothalamic nucleus (PVH). Acute restraint increased the number of PVH cells expressing Fos, with greater increases in males than females. Habituated responses were seen following repeated stress in both sexes, with no sex differences between groups. No sex differences were found in the number of neurons co-expressing Fos and AVP. Absolute counts of cellular Fos and GR co-localization mirrored Fos expression. In contrast, when doubly-labeled cells were normalized to staining for Fos alone, females showed greater numbers of Fos- and GR-positive cells than males after both acute and repeated stress. These data demonstrate that sex-specific stress responses are evident at the level of neuronal activation, and may contribute to different consequences of chronic stress in females versus males. Females may be more sensitive to glucocorticoid negative feedback, suggesting that sex-dependent differences in the efficiency of initiating and terminating stress responses may exist. Understanding the neural and endocrine pathways that mediate these functions in males and females will inform targeted therapeutic strategies to alleviate stress and the sex-specific afflictions with which it is associated.



## 1. Introduction

"Stress" is a term that can be used to describe a wide variety of stimuli, both physiological and psychological, that can have major direct and indirect effects on bodily function [1]. Acute or short-term stress has been shown to induce an array of neurological and endocrine responses geared toward survival and the maintenance of homeostasis. Repeated or chronic stress, in contrast, often leads to an adaptation of these responses, yet has been linked with the onset or increased severity of numerous diseases including affective, immune and cardiovascular disorders (for recent review, see [2]). It is critically important, therefore, to understand how stress responses are initiated, and ultimately terminated, in order to effectively identify targets for the treatment of these stress-related illnesses which are becoming increasingly pervasive, contribute significantly to the rising costs of healthcare, and negatively impact quality of life in those who suffer from these disorders. Furthermore, differential responses to stress have been observed in males and females, and sex-specific outcomes have been noted in terms of stress-related disease incidence, but the mechanisms through which these outcomes are mediated remain unclear. The present studies were thus undertaken to more clearly define the central nervous system pathways and mechanisms that are activated by stress, with particular attention given to how these factors may differ in females compared to males since sex is one of the most important predictors of health. As a consequence of chronic stress, women tend to suffer more often from autoimmune illnesses while men are at a greater risk for developing coronary or infectious diseases [3]. With regard to psychiatric disorders, women more often experience anxiety, depression or panic disorders, while substance abuse and antisocial behaviors are more common in men [3]. Understanding the biological bases of these differences will inform future efforts to prevent or treat these sex-specific pathologies [4].

The hypothalamic–pituitary–adrenal (HPA) axis is a neuroendocrine control and regulatory mechanism through which the central nervous system can modulate peripheral hormone secretion and physiological function. Activated by stress, the HPA axis involves the secretion of corticotropin-releasing factor (CRF) from the paraventricular hypothalamic nucleus (PVH), which then stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH, in turn, induces glucocorticoid (GC) release from the adrenal cortex. The PVH, therefore, is responsible for the

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generation of stress responses as it lies at the head of the HPA axis. Several studies have demonstrated that the PVH is activated by stress, as evidenced by enhanced expression of CRF mRNA or protein, c-fos mRNA, or Fos protein [5–8]. Furthermore, neuronal activation levels in the PVH have been shown to be high during acute stress, but decrease or habituate in response to repeated homotypic stress exposure [6,9].

Cells that remain active in the repeated stress condition tend to be localized within a more ventral subregion of the PVH, compared to the more dorsally situated PVH neurons which are seen to be activated during acute stress [10]. This suggests that distinct subsets of neurons in the PVH may exist, and may be differentially responsible for initiating stress responses and maintaining them in the repeated condition. We therefore investigated Fos expression in the PVH in response to acute and repeated restraint stress, and further determined whether the activated neurons in each case express distinct neuropeptide phenotypes that would allow us to discriminate their function.

Arginine vasopressin (AVP) is an osmoregulatory neuroendocrine hormone which is normally secreted by magnocellular neurons in the PVH. During prolonged stress, however, AVP is also increasingly expressed and co-released from parvocellular PVH neurons, and travels to the pituitary to act as a co-secretagogue with CRF to enhance ACTH release [11]. AVP is believed to play an important role in sustaining pituitary responsiveness during chronic stress [12], and CRF and AVP are both essential for coordinating the behavioral and metabolic responses to stress [13]. Circulating GCs bind to glucocorticoid receptors (GRs), low-affinity corticosteroid receptors that are expressed at high levels throughout the brain and pituitary [14]. A member of the nuclear hormone receptor family of ligand-activated transcription factors [15], GRs are active when GC concentrations are high, and serve to mobilize energy resources and terminate stress responses through negative feedback at the level of the hypothalamus [13]. GRs also facilitate recovery from stress by interfering with transcription and repressing cellular activities that are induced by stress such as increased CRF and AVP synthesis [13]. In addition, GCs are capable of binding to GRs in extrahypothalamic brain regions that can subsequently modify the activity of the HPA axis [16]. The secretion of GCs into the circulation is primarily mediated by the HPA axis [16]; inhibition of this axis serves to minimize the amount of tissue exposure to GCs, thus reducing the catabolic, lipogenic, anti-reproductive and immunosuppressive effects of these hormones [16]. Lastly, GR promotes memory storage in preparation for future stressful events [13].

Given the highly significant roles of AVP and GR in HPA axis regulation, we examined the PVH in male and female rats under control, acute stress and repeated stress conditions to determine if stress-sensitive neurons would also express these markers. Our hypothesis was that the activation and phenotype of hypothalamic neurons that function in the initiation and termination of stress responses would differ by stress condition and sex. We further anticipated that the mechanisms through which sex-specific responses to stress are mediated would include neuroendocrine and feedback control systems involved in fluid and energy homeostasis, as these mechanisms are critical for the survival of females and their offspring.

## 2. Methods

### 2.1. Experimental animals

Young adult (3–4 months of age) male and female Sprague Dawley rats (Harlan, Houston, TX) were used in the present experiments. All rats were individually housed in standard cages in a temperature-controlled animal facility maintained on a 12:12 h light:dark cycle, with food and water provided ad libitum. Following shipment, the rats were allowed at least 1 week of acclimatization to the facility before experimentation was initiated. Rats were randomly assigned to Control, Acute restraint or Repeated restraint groups (n = 5/group). Animal care and use were in accordance with the Guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee.

### 2.2. Restraint stress

Emotional stress was applied in the form of acute or repeated restraint, which consisted of placing the rats in a plastic restraining device (Kent Scientific, Torrington, CT) for 30 min. Repeatedly stressed rats were restrained in their home cages for 30 min daily over 14 consecutive days. Acutely stressed rats were exposed to open restrainers for 30 min/day over the first 13 days, then restrained for 30 min on the 14th day only. Unstressed control rats were exposed to open restraining devices on each of 14 consecutive days, but were never restrained. All restraint and exposure occurred near the beginning of the light cycle, between the hours of 0900 and 1100.

### 2.3. Perfusion

Rats were transcardially perfused 2 h after the termination of restraint or exposure on the final day. Rats were weighed and deeply anesthetized by i.p. injection of 100 mg/kg sodium pentobarbital (Nembutal®; McKesson, Washington Courthouse, OH). Perfusion through the ascending aorta was done using ~100 mL of ice-cold 0.9% saline, followed by 400–500 mL of ice-cold 4% paraformaldehyde (JTBaker, Inc., Pittsburg, NJ) at pH 9.5 in 0.1 M borate buffer. Brain tissues were then collected and post-fixed for 5 h at 4 °C, followed by cryoprotection overnight at 4 °C in 10% sucrose in KPBS.

### 2.4. Tissue processing

The following day, brains were removed from cryoprotectant, mounted on a tabletop freezing microtome (Model SM 2000R; Leica Microsystems, Bannockburn, IL), and serial frozen sections taken in the coronal plane from a block of brain tissue containing the hypothalamic paraventricular nucleus. Five 1:5 series at 30 μm intervals were collected into antifreeze (30% ethylene glycol, 20% glycerol) and stored at −20 °C until used for immunohistochemical analyses.

### 2.5. Immunohistochemistry

Brain tissue sections were immunohistochemically stained for peptides and receptors known to play key roles in the stress response. First, Fos immunoreactivity was measured using a nickel-intensified avidin–biotin-immunoperoxidase technique. Sections were washed in KPBS, then placed in 0.3% hydrogen peroxide to quench endogenous peroxidases and 1% sodium borohydride to reduce free aldehydes. After being washed thoroughly, the tissue was placed in primary antiserum (rabbit anti-Fos; Oncogene Science, Cambridge, MA) diluted 1:50,000 in KPBS containing 0.3% Triton X-100 and 2% normal goat serum and incubated at 4 °C overnight with gentle agitation. On the following day, sections were incubated in secondary antibody (biotinylated goat anti-rabbit IgG, 1:200 dilution; Vector Laboratories, Burlingame, CA) for 1 h. An avidin–biotin-complexing solution (Vectastain Elite kit; Vector Laboratories, Burlingame, CA) was then applied for 1 h, and a nickel-enhanced glucose oxidase method using diaminobenzidine (DAB) as a chromogen was utilized to visualize specific binding [17].

Subsequently, dual localization of Fos with other markers was accomplished using the same immunoperoxidase method as described above but with sequential staining for the second marker done without nickel enhancement. Cells expressing both markers were therefore visible as having black nuclei (Fos) and a brown cytoplasm