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# Stimulation of the prelimbic cortex differentially modulates neuroendocrine responses to psychogenic and systemic stressors

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ABSTRACT

The medial prefrontal cortex is important for normal regulation of stress responses, and is implicated in stress-related affective disease states (e.g. depression). In the current study, we investigated the role of the prelimbic division of the prefrontal cortex in control of responses to psychogenic and systemic stressors (restraint and hypoxia, respectively). Acute stimulation of the prelimbic cortical region with bicuculline methiodide (BMI) caused significant reduction of ACTH and corticosterone responses to restraint and reduced Fos activation of paraventricular nucleus neurons, consistent with a role in central inhibition of acute psychogenic stress responses. In contrast, BMI enhanced corticosterone (but not ACTH) responses to hypoxia via a mechanism suggestive of central PVN drive and enhanced adrenal sensitivity. Acute BMI increased restraint stress-induced Fos activation in known downstream targets of the prelimbic cortex (e.g., the basolateral amygdala and central amygdaloid nuclei), suggesting a connection between modulation of amygdalar signaling and stress inhibition. In contrast, hypoxia caused robust Fos activation in the basolateral and central amygdala, which was not affected by prelimbic BMI injection. The data suggest that the prelimbic cortex stimulation is sufficient to trigger inhibition of the HPA axis to psychogenic stress, but may play a very different role in enhancing HPA responsiveness to physical threats.



## 1. Introduction

The mammalian prefrontal cortex (PFC) is required for appropriate processing of stressful information. In humans, prefrontal cortex dysfunction is linked to stress-related diseases such as PTSD and depression [1,2]. Prefrontal cortex complex volume is associated with glucocorticoid regulation in man [3], suggesting a functional link between the PFC and regulation of stress hormone secretion.

Recent studies reveal that the role of the PFC in stress regulation is complex, with different subregions having divergent actions on stress responses. In rodents, lesions of the dorsal divisions of the medial PFC, largely targeting the prelimbic cortex (PL), result in prolonged glucocorticoid responses to stressors of psychogenic (e.g., restraint, air puff), but not systemic (e.g., ether) origin [4–6]. Enhanced corticosterone responses are associated with increased Fos activation in hypophysiotrophic corticotrophin-releasing hormone neurons of the hypothalamic paraventricular nucleus [6,7], consistent with enhanced drive of the neural limb of the hypothalamo–pituitary–adrenocortical (HPA) axis. Basal corticosterone release is not affected by PL lesion [4,5,8], suggesting that the PL primarily affects stress activation of the HPA axis. Notably, inhibition of the PL enhances heart-rate responses to psychological stimuli [9], suggesting that PL activation also controls autonomic stress responses. In contrast, lesions of the extreme ventromedial division of the PFC (encompassing the infralimbic cortex (IL)) result in slight attenuation of HPA axis stress responses [7,10], and lesion or inactivation of this region reduces cardiovascular responses to psychogenic stress [9]. Thus, the dorsal and ventral divisions of the PFC (PL and IL, respectively) appear to have very different and perhaps opposing roles in stress regulation.

Neither the PL nor the IL project directly to the paraventricular nucleus of the hypothalamus (PVN) [11–13]. As is the case with other limbic HPA-regulatory structures (e.g., hippocampus, amygdala) (see [14]), the two PFC divisions act on the PVN via intermediary synapses. Targets of the PL include PVN-projecting regions such as the bed nucleus of the stria terminalis (BST) [6], lateral hypothalamus (LHA) and posterior hypothalamus. The PL also projects heavily to the paraventricular thalamus (PVT) [15], which is known to play a prominent role is stress habituation and sensitization [13]. In the case of the BST, at least a proportion of the PL-PVN relays are GABAergic [16], consistent with trans-synaptic inhibitory actions of the PL on HPA axis stress responses. The IL also targets the BST and LHA, and also projects to additional potential PVN relay sites in nucleus of the solitary tract and dorsomedial hypothalamus [6,11–13]. In addition to targeting PVN projecting regions, the PL heavily innervates the basolateral amygdala (BLA) [12], which is known to modulate HPA axis function via trans-synaptic relays [14,17].

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The current study was designed to test the sufficiency of the PL for stress circuit activation and HPA axis regulation in vivo. Microinjections of the GABA-A antagonist bicuculline methiodide (BMI) were used to acutely activate the PL. Our data support an inhibitory role for the PL in regulation of responses to acute psychogenic stress, but also suggest an opposite role in mediating responses to systemic challenge.

## 2. Methods

### 2.1. Animals

Male Sprague–Dawley rats (250–300 g; Harlan, Indianapolis, IN) were housed individually in standard rat cages and acclimated for 1 week prior to initiation of experiments. Rats were maintained in a temperature- and humidity-controlled room (lights on 06:00 to 18:00) with food and water available *ad libitum*. All experimental procedures and protocols were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the University of Cincinnati Institutional Animal Care and Use Committee.

### 2.2. Cannula placement and microinjections

Animals were anesthetized a mixture of ketamine (87 mg/kg) and xylazine (13 mg/kg). Double-barrel guide cannulae (Plastics One, Roanoke, VA) were stereotaxically implanted (+3.0 mm from bregma, 0.75 mm left and right of midline, and 3.0 mm below the skull) so that the tips of the cannulae were 0.5 mm superior to the PL. Cannulae were secured by dental cement and sealed with a double dummy cannula cut to the same dimensions. Rats were handled daily for 2 weeks and cannulae were manipulated in order to habituate animals to the injection procedure. On the day of injection, dummy cannulae were removed and replaced with internal injector cannulae under light manual restraint. Initial studies employed conjoint unilateral infusions of BMI and saline (performed simultaneously using a Harvard microsyringe pump) in unstressed rats to estimate extent of Fos activation by BMI. For restraint and hypoxia stress studies, bilateral infusions of BMI or vehicle were performed simultaneously using a Harvard microsyringe pump, with a total volume of 500 nl delivered over 60 s. Both BMI (1 ng/μl) and saline were co-injected with Pontamine Sky Blue (1%) to mark the site of injection and approximate spread of injectate. Injections were performed immediately before initiation of restraint or hypoxia.

### 2.3. Stress protocols

Stress testing was initiated between 09:00 and 10:00, during the circadian trough of CORT secretion. Different groups of injected animals were exposed to either acute restraint, serving as a psychogenic stressor, or hypoxia, serving as a systemic stressor, with plasma CORT and ACTH responses used as indices of HPA activation. A basal blood sample was taken by tail clip immediately prior to stress exposure in all rats. For restraint, animals were placed in well-ventilated Plexiglas restraint tubes for 30 min and tail clip blood samples (200 μl) were collected immediately prior to removal from the restrainer and 30 and 90 min after being returned to their home cages. For hypoxia, rats were placed in the testing chamber with a 8% oxygen/92% nitrogen mixture permeated through the chamber for 30 min. Blood was sampled by tail clip under light manual restraint 30, 60 and 120 min following initiation of hypoxia.

### 2.4. Blood collection and radioimmunoassay

Blood was sampled by the tail clip procedure that involves clipping the tail with a sterile scalpel blade and collecting blood in 1.5 ml microcentrifuge tubes containing 10 μl 100 mM EDTA. Tubes were then spun at 1500 g and plasma pipetted into 0.5 ml tubes and frozen at −20 °C for subsequent analysis of plasma CORT and ACTH. Plasma CORT levels were measured using a $^{125}$I RIA kit (MP Biomedicals Inc., Orangeburg, NY). Plasma ACTH concentrations were determined with an RIA that used a specific antiserum donated by Dr. William Engeland (University of Minnesota, Minneapolis, MN) at a dilution of 1:120,000 with $^{125}$I ACTH (Amersham Biosciences, Piscataway, NJ) as a labeled tracer. For each assay, all plasma samples were run in duplicate and analyzed within the same assay.

### 2.5. Tissue collection and immunohistochemistry

Two hours after initiation of restraint or hypoxia, rats were given an overdose of sodium pentobarbital and perfused transcardially with 0.9% saline followed by 3.7% paraformaldehyde in 0.1 M PBS. Brains were removed, postfixed in 3.7% paraformaldehyde overnight at 4 °C, and then placed in 30% sucrose dissolved in PBS. Brains were serially sectioned at 25 μm on a freezing microtome and stored at −20 °C in sterile cryoprotectant solution until processing. For immunohistochemistry, sections were rinsed several times in 50 mm potassium PBS (KPBS; pH 7.4) to remove cryoprotectant. Sections were incubated in 1% $H_2O_2$ to quench endogenous peroxidase followed by washing in KPBS (5×5 min). Sections were subsequently blocked for 1 h with 0.2% bovine serum albumin in 50 mm KPBS with 0.2% Triton-X 10. Sections were incubated overnight at 4 °C in rabbit polyclonal anti-Fos (dilution 1:20,000; Calbiochem, San Diego, CA, USA). The next day, sections were washed in KPBS (5×5 min), incubated in biotylinated goat anti-rabbit (dilution 1:500) (Vector Laboratories, Burlingame, CA, USA) for 1 h, washed in KPBS (5×5 min), and reacted with avidin–biotin peroxidase (dilution 1:800) (ABC Elite Kit; Vector Laboratories) for 1 h. Sections were washed once again in KPBS (5×5 min) and subsequently reacted with 0.02% diaminobenzidine/0.09% hydrogen peroxide in KPBS. Reactions were allowed to proceed for 5–10 min, at which point the reaction was stopped by sequential rinses in KPBS. Sections were mounted on gelatinized slides, allowed to dry, dehydrated with alcohol and xylene, and coverslipped.

### 2.6. Cell counting

Digital images were collected for quantification of Fos positive immunoreactive nuclei in regions of interest. The number of Fos-immunoreactive cell nuclei was determined from thresholded images using Scion Image software. A uniform threshold (based on a predefined threshold function in Scion Image) was applied to all images in a given region of interest and the average cell count was automatically calculated. The final cell counts were expressed as the mean number of positive nuclei. The shape and size of each region of interest studied were defined according to the boundaries outlined in Paxinos and Watson (1998). A total of 2–6 images were analyzed for each region and averaged to produce a mean cell count for each region.

### 2.7. Data analysis

Time-course data for ACTH and CORT were analyzed using two-way repeated measures ANOVA with treatment and time as factors. Treatment differences at individual time points were assessed using Fisher's LSD post-test. Area under the curve (AUC) for ACTH and CORT responses to restraint and hypoxia were analyzed with the t-test. Adrenal sensitivity was estimated as the concentration of CORT divided by the log of the concentration of ACTH [18]. Differences in Fos cell counts between treatments were determined by t-test.