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Sexual dimorphism in locus coeruleus dendritic morphology: A structural basis for sex differences in emotional arousal

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ABSTRACT

Stress-related psychiatric disorders, such as depression and anxiety, affect a disproportionate number of women. We previously demonstrated that the major brain norepinephrine (NE)-containing nucleus, locus coeruleus (LC) is more sensitive to stressors and to the stress-related neuropeptide, corticotropin-releasing factor (CRF) in female compared to male rats. Because the LC–NE system is a stress-responsive system that is thought to be dysregulated in affective disorders, sex differences in LC structure or function could play a role in female vulnerability to these diseases. The present study used different approaches to compare LC dendritic characteristics between male and female rats. Immunofluorescence labeling of tyrosine hydroxylase, the norepinephrine synthetic enzyme, revealed that LC dendrites of female rats extend further into the peri-LC region, covering a significantly greater area than those of males. Optical density measurements of dendrites in the peri-LC revealed increased dendritic density in females compared to their male counterparts. Additionally, immunoreactivity for synaptophysin, a synaptic vesicle protein, was significantly greater in the LC in female rats, suggesting an increased number of synaptic contacts onto LC processes. Individual LC neurons were juxtacellularly labeled with neurobiotin *in vivo* for morphological analysis. LC dendritic trees of females were longer and had more branch points and ends. Consistent with this, Sholl analysis determined that, compared to males, LC dendrites of females had a more complex pattern of branching. The greater dendritic extension and complexity seen in females predicts a higher probability of communication with diverse afferents that terminate in the peri-LC. This may be a structural basis for heightened arousal in females, an effect which may, in part, account for the sex bias in incidence of stress-related psychiatric disorders.

1. Introduction

Stress-related psychiatric disorders such as depression, anxiety and post-traumatic stress disorder (PTSD) are more prevalent in women compared to men, and this has been attributed to greater sensitivity of stress-responsive systems [1–7]. One stress-response system that could account for sex differences in the prevalence of stress-related psychiatric disorders is the locus coeruleus (LC)–norepinephrine (NE) system. The nucleus LC is a major source of NE in the brain and the sole source of NE in hippocampus and cortex [8,9]. The LC–NE system mediates arousal, attention, and vigilance in response to salient stimuli [10–12]. Additionally, LC neurons are activated by stressors, resulting in NE release throughout the forebrain [13–15]. This is thought to mediate an emotional arousal limb of the stress response that is coordinated with behavioral, autonomic and endocrine components of the stress response [16,17]. Although the LC response to acute stress is adaptive, if it extends beyond the duration of the stressor or if it occurs in the absence of stress this could be expressed as pathological hyperarousal or anxiety. LC dysregulation has been hypothesized to occur in conditions of chronic stress and has been implicated in melancholic depression and the hyperarousal and re-experiencing symptoms of PTSD [18–22]. Because these disorders occur more frequently in women than in men, it is possible that sex differences in the structure or activity of the LC–NE system may predispose women to stress-related disorders.

One previously documented sex difference in LC structure was a larger LC composed of more neurons in female Wistar rats compared to males [23,24]. This sex difference resulted from an increase in postpubertal proliferation of LC neurons in females and was dependent on gonadal hormones [25]. Females require a lifelong presence of estradiol for a feminized LC, whereas males require a perinatal testosterone surge for LC masculinization [23,25–27]. However, the finding of sex differences in LC volume did not generalize to all strains or species [28–31].

More recently, we demonstrated sex differences in LC neurons at a functional level [32,33]. Stress produced a greater activation of LC neurons in female rats and these neurons were more sensitive to the stress-related neuropeptide, corticotropin-releasing factor (CRF), which is thought to mediate activation of the LC–NE system during

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stress [17,32–34]. In addition to increasing LC neuronal activity, CRF affects dendritic architecture, increasing dendritic length of LC neurons in slice cultures and promoting neurite outgrowth in LC-like CATHa cells [35,36]. This structural effect could further influence stress vulnerability by increasing the probability of communication with brainstem and limbic afferents conveying autonomic and emotionally-related information to the LC.

The present study was designed to compare different characteristics of LC dendrites between adult male and female rats. The majority of LC inputs innervate dendrites that extend into the pericoerulear region (peri-LC) [37]. Therefore, we first determined the extent and density of LC dendrites in the peri-LC using immunofluorescence labeling for tyrosine hydroxylase (TH), the norepinephrine synthetic enzyme. To compare dendritic morphology in more detail, individual LC neurons of male and female rats were juxtacellularly labeled with neurobiotin and dendritic architecture was analyzed and compared between groups.

2. Methods

2.1. Subjects

Adult (65–85 days old) male and female Sprague–Dawley rats (Charles River, Wilmington, MA) were used. All rats had *ad libitum* access to food and water, were maintained on a 12 h light/dark cycle, and housed in same sex groups (2–3 rats/cage). For cycling females, vaginal cytology was used to track the estrous cycle as previously described [38]. For the initial study, which examined TH and synaptophysin immunoreactivity, half of the females were perfused in diestrus (characterized by low estrogen levels) while the other half were perfused in proestrus (characterized by high estrogen levels) to ensure that both hormonal extremes were equally represented. Care and use of animals was approved by the Children's Hospital of Philadelphia's Institutional Animal Care and Use Committee and in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Immunohistochemistry for densitometry

Rats were transcardially perfused with saline 0.9% followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). After perfusion, brains were removed, post-fixed for at least 1 h, and then stored in a 20% sucrose solution in PB with 0.1% sodium azide at 4 °C for at least 48 h. Frozen coronal sections (30 μm) were cut through the LC on a cryostat and stored in either PBS for immediate processing or in cryoprotectant for long term storage.

For TH and synaptophysin immunoreactivity, every fourth section was first incubated in 0.75% H_2O_2 for 20 min and then rinsed in phosphate buffered saline (PBS) containing 0.3% Triton and 0.04% bovine serum albumin (PBS-TX-BSA) three times for 30 min. Sections were then incubated in PBS-TX-BSA and 0.1% sodium azide for 48 h at 4 °C with either mouse anti-TH (1:5000, Immunostar) or rabbit anti-TH (1:2000, Millipore) and mouse anti-synaptophysin (1:75,000, clone SY38, Millipore). Specificity of the anti-synaptophysin antibody has been characterized previously using a preabsorption control [39]. Sections incubated in anti-TH antibody alone were rinsed (PBS-TX-BSA) three times over 30 min and incubated in donkey anti-mouse antibody conjugated to Alexa Flour 488 (1:200, Invitrogen) for 90 min at room temperature. For dual labeling of TH and synaptophysin, sections were rinsed as described, then incubated with donkey anti-mouse antibody conjugated to Alexa Flour 594 and donkey anti-rabbit antibody conjugated to Alexa Flour 488 (1:200, Invitrogen) for 90 min at room temperature. Sections were then rinsed in PB, mounted, and cover-slipped with Fluoromount G (SouthernBiotech, Birmingham, AL, USA). Immunoreactivity was visualized by fluorescence microscopy using a Leica DMRXA microscope. Images were captured with a Hamamatsu ORCA-ER digital camera (Bridgewater, NJ, USA) using Open Laboratory software (Improvision, Coventry, UK).

2.3. Quantification of dendritic area and density

Coronal sections were selected from images of TH staining that corresponded to a rostral and mid rostrocaudal level of the LC as shown in Fig. 1A. These are comparable to the zones previously described as those in which the majority of LC processes extend [40]. Previous electron microscopic studies demonstrated that nearly all of the labeled processes in these regions are dendrites [40]. Fluorescent images were taken at the same exposure, then converted to grayscale and inverted. The Image J Optical Density calibration was used to determine dendritic density in triangular regions of interest in both ventromedial and dorsolateral peri-LC regions (Fig. 1A). In addition to the density measurements, the area covered by LC dendrites in ventromedial peri-LC was outlined using Image J, and measurements were converted from pixels^2 to μm^2 for graphical presentation. To evaluate potential sex differences in TH expression, the optical density of TH in the nuclear core of the mid LC region, which contains the most cell bodies, was analyzed by outlining the portion of the LC containing only the cell bodies. For all density measurements, the mean density of a background region (taken from a portion of the image with little TH staining) was subtracted from the mean density of the region of interest and the result was multiplied by the area of the region of interest to yield the integrated density measurement. For rats with multiple rostral or mid LC sections, the data from the multiple sections

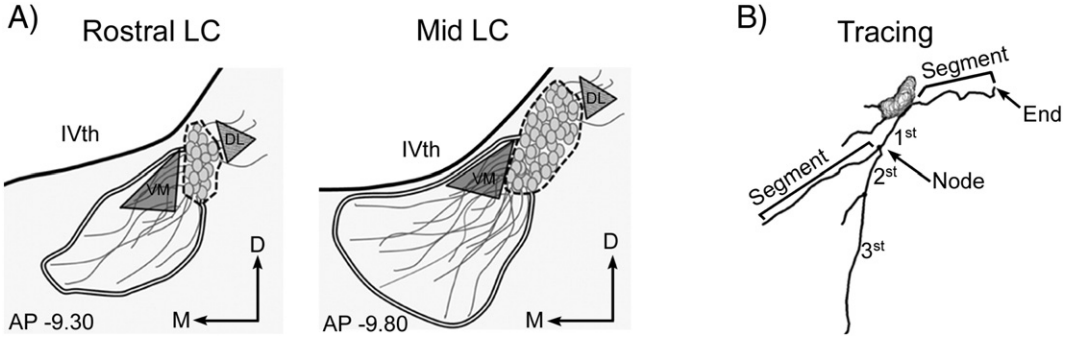

Fig. 1. Diagrams depict endpoints analyzed for densitometry and morphology. (A) Schematics represent the regions sampled in a level of the rostral LC (left) and a middle level of the rostrocaudal LC (right). The rostral and mid LC regions are AP −9.30 and −9.80 relative to Bregma, respectively [62]. The nuclear core (i.e., LC cell bodies) is defined by the dashed line. The double line demarcates the area of the ventromedial dendrites analyzed. The shaded triangle represents the region of interest used to assess the density of the ventromedial dendrites. The striped triangle represents the region of interest used to assess the density of the dorsolateral dendrites. (B) Shown on the representative trace are examples of an end, a node, and segments. This neuron also had primary (1st), secondary (2nd), and tertiary dendrites (3rd). Abbreviations: IVth, fourth ventricle; D, dorsal; DL, dorsolateral; M, medial; VM, ventromedial.