Early life stress induces sex-specific changes in behavior and parallel locus coeruleus neuron excitability

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Abstract

Stress-related psychiatric disorders are more common in female clinical populations after adolescence, but the exact mechanism behind this difference is unknown. The locus coeruleus (LC) is a brain area that contains adrenergic norepinephrine (NE)-releasing neurons previously found to display basal sex differences in excitability and stress neuropeptide responses in animal models. Corticotropin releasing factor (CRF), the neuropeptide responsible for stress signaling, has been shown to directly alter LC neuron firing, thereby providing one mechanism by which stress may modulate noradrenergic function in either acute or chronic stress. Such activation of CRF receptor 1 (CRF_1) by CRF in the LC is fundamentally different in male and female mice due to increased CRF receptor expression, G-protein coupling, and CRF-stimulated NE release in female compared to males. Despite evidence for an interaction between sex, LC adrenergic neuron activity, and stress, how early life stress affects LC neuronal behavior in parallel to anxiety, activity, and cognition (i.e. components of PTSD and ADHD) in a sexual dimorphic manner has not been explored. The effects of an early life variable stress (ELVS) paradigm inducing stress at both early life and adolescence were assessed in C57 male and female mice using behavioral and electrophysiological measures for hyperactivity, anxiety, short-term memory, repetitive behaviors and LC neuron excitability. Hyperactivity in open field and deficits in Y-maze performance were observed in ELVS female mice when compared to controls. ELVS females, but not males, showed a reduction in LC excitability when compared to controls of the same sex, which was due to hyperpolarization and an increase in the action potential delay time. LC neurons form naïve mice indicated that CRF_1 receptor-mediated effects on LC neuron excitability were largely due to G_q-mediated (Ca^{2+}-dependent) signaling in male mice and non Ca^{2+}-dependent (likely G_s) in female mice. Through this animal model of early life stress inducing impaired memory and hyperactivity in a sex-specific manner, further investigation to ameliorate these symptoms may benefit more targeted treatment approaches for stress-related disorders based on sex and developmental stage of trauma exposure(s).

Introduction

According to the DSM-IV there are several types of anxiety disorders that include post-traumatic stress disorder (PTSD), with memory deficits and difficulty concentrating key features shared among anxiety disorders (1). Although mature neurological systems are often resistant to acute stress, prolonged periods of stress in more malleable and immature systems can alter neurodevelopment. Epidemiological studies indicate women may have increased stress-related disorder development risk/symptom exacerbation during life-phases characterized by hormonal changes that include puberty, menses, pregnancy, postpartum, and menopause (2,3). We were particularly interested in how the adolescent time point may increase stress sensitivity in females impacting developmental stress-related disorder symptoms.

CRF receptor activation directly alters activity of adrenergic neurons releasing norepinephrine (NE) in the locus coeruleus (LC)–a key brain region responsible for shaping arousal, aversion, memory and attention (4–6). Optimal LC neuron activity and related NE release in the prefrontal cortex, amygdala, and hippocampus shape attention, anxiety, and memory (7–11). In an animal model of trisomy 21, LC degeneration is observed along with hyperactivity and impaired spatial and working memory, with these behaviors ameliorated by increasing LC neuron activation using Gq-coupled DREADDs (12). Animal model data show that compared to males, females have elevated CRF sensitivity due to increased CRF receptor expression resulting in increased basal NE release from the LC neurons (12–14). These basal sex differences may underpin females' increased stress-related disorder prevalence (15,16). Despite the established role of NE in attention and anxiety along with established sex differences in LC and NE dynamics, how early stress interacts with LC sex differences to shape behaviors associated with LC function (i.e. anxiety, cognition, and locomotion) is poorly understood. To identify and understand the mechanistic relationship between sex and affective, cognitive, and locomotive behaviors associated with stress, we developed a two-hit model of stress exposure early in life to assess if these behaviors were affected and if there were sex-specific LC physiology changes. Our results

suggest an interaction between sex and stress in cognitive and locomotive behaviors that may involve the LC.

Methods and Materials

Animals

Male and female C57bl6/J (Jackson Labs, #000664) or Dbh-tdTomato mice (described below) were purchased from Jackson Laboratory and/or bred in house. To generate mice expressing tdTomato in locus coeruleus noradrenergic neurons under control of dopamine beta hydroxylase (Dbh-tdTomato mice), Dbh-Cre knock-in mice (Dbh$^{tm3.2(cre)Pjen}$/J; stock #033951; Provided by Patrician Jensen, NIEHS) were crossed with homozygous Ai14 tdTomato reporter mice (B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG\text{-}tdTomato)Hze}$/J, Stock #007914, provided by Hongkui Zeng, Allen Institute for Brain Science). All mice were group housed under a reversed 12-hour light-dark cycle (lights on from 21:00 to 9:00) with *ad libitum* access to food and water. All manipulations and tests were performed during the animals' active dark phase (9:00 to 21:00), unless otherwise stated. All procedures involving animals were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee (#2022-100) of Southern Illinois University - School of Medicine.

Early Life Variable Stress (ELVS) Animal Model

Male and female C57bl6/J mice were subject to either a two-hit (postnatal and adolescent) early life variable stress (ELVS) paradigm or treated as controls and all mice in a litter were either exposed to ELVS or treated as controls. The ELVS paradigm consisted of two phases of manipulations, first from postnatal day 6-17 and the second from postnatal day 28-40. First, mice in the ELVS group were isolated from dams for 4hrs/day during postnatal day 6-10 (P6-10) and weaned early at postnatal day 17 (P17). Pups were supplemented with nutrient gel (Nutra-gel from Bio-serve) from P17 to P21. ELVS mice were subjected to ten consecutive days of stressors

with one stress exposure per day from P28-42 with the order of the five stressors varied randomly between cohorts. These stressors included 20 minutes of tape restraint (2x), 20 minutes of 2,3,5-Trimethyl-3-thiazoline (TMT) synthetic fox odor exposure (2x), 3 minutes of forced ice-cold swim in a 40 cm x 10 cm filled container (2x), 36 hours of light cycle disruption via continueous light exposure (1x), and 48 hours of social isolation (1x). Control male and female C57bl6/J mice were not separated from dams and weaned at P21 and were briefly handled 5 times per week for 10-15 minutes beginning at the same time as the stressors (P28) for the ELVS group.

Behavioral Tests

Behavioral tests were done during the animals' active dark phase in a ~15-20 lux dimly lit room for testing. Mice were habituated to the testing room for 30 minutes prior to running the experiments. Behavioral assays were recorded with a system for video tracking. Each apparatus was cleaned thoroughly with scent-free disinfectant between animals.

Open Field Test

Mice were placed in a white Plexiglas chamber (40x40cm square open top chamber that is 30cm high) for 30 min with total distance, time in center relative to edges and center entrances calculated using the EthoVision XT 17 software (Noldus).

Y-Maze

Mice were placed in a 3-armed "Y" shaped apparatus with symmetrical arms 35 cm in length, 30 cm wall height and 10 cm lane widths at 120-degree angles from one another. Total alternations, or the number of times the mouse enters 3 arms, were recorded and compared to correct alternations where every arm entered within the 3 entrances had not been entered in the immediate previous entrance.

Elevated Zero Maze

Mice were placed in the open arm of an elevated circular platform, with 2 parallel walled sections and 2 open sections in between. The amount of time spent in the open versus closed segment along with open arm entrances were calculated using Ethovision XT 17.

Marble Burying

A standard mouse cage with 3 cm of fresh bedding was used with 16 glass marbles placed in a grid pattern with the number of marbles at least 75% covered counted at 5-minute intervals over a 30 min period.

Ex vivo Acute Brian Slice Preparation

Animals were anesthetized with 4% isoflurane, followed by intracardial perfusion with ice-cold oxygenated N-methyl-d-glucamine (NMDG) artificial cerebrospinal fluid (NMDG-ACSF), which contained (in mM): 92 NMDG, 2.5 KCl, 0.5 CaCl2, 10 MgCl2, 1.2 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 d-glucose, 2 ascorbic acid, 2 thiourea, and 3 sodium pyruvate and had an osmolarity of 300–310 mOsm with pH adjusted to 7.3–7.4 with HCl. Coronal slices that included the LC were taken at 250 μm, sectioned using ice-cold NMDG-ACSF with a Compresstome vibrating microtome (Processionary Instruments, LLC) and were transferred to a holding chamber containing NMDG-ACSF at 35 °C where the NaCl concentration increased steadily over 25 min. After 30 min, slices were transferred to a modified HEPES-based ACSF (HEPES-ACSF, 35 °C), which contained (in mM): 92 NaCl, 2.5 KCl, 2 CaCl2, 2 MgCl2, 1.2 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 d-glucose, 2 ascorbic acid, 2 thiourea, 3 sodium pyruvate, and 3 myo-inositol and had an osmolarity of 300–310 mOsm with pH 7.3–7.4. After incubating slices in HEPES-ACSF at 35 °C for 1 hr, slices were maintained in HEPES-ACSF at room temperature until being transferred to the recording chamber where they were continuously perfused at a 3-5 ml/min with oxygenated ACSF, which contained (in mM): 125 NaCl, 2.5 KCl, 2 CaCl2, 2 MgCl2, 1NaH2PO4, 26 NaHCO3,

20 D-glucose, 2 ascorbic acid, and 3 myo-inositol and had an osmolarity of 310–320 mOsm with pH 7.3–7.4.

Ex vivo Acute Brain Slice Whole-Cell Electrophysiology

Neurons in the LC were visualized using an upright microscope (Olympus BX51WI) with a 40x water immersive objective. The microscope was able to detect interference contrast and contained an infrared camera connected to a video monitor. Whole-cell patch-clamp recordings were performed at 32 °C maintained with an in-line solution heater from visually identified LC neurons using the 4th ventricle as a landmark. Noradrenergic neurons in the LC were identified by the presence of tdTomato in Dbh-tdTomato mice and action potential properties of putative noradrenergic LC neurons in C57bl6/J mice were verified to be similar to Dbh-tdTomato+ neurons.

Using a horizontal puller (P1000, Sutter Instruments), patch pipettes were pulled from filamented borosilicate glass capillaries (outer diameter 1.5 mm, inner diameter 0.86, Sutter Instruments), having a tip resistance of 5–7 MΩ when filled with potassium gluconate-based internal solution with an osmolality 285–290 mOsm and the pH adjusted to 7.2-7.3 with KOH. All signals were acquired at 20 kHz and low-pass filtered at 10 kHz via a MultiClamp 700B amplifier. Data were collected from the neurons with an input resistance >100 MΩ. If the series resistance for a given recording was >30 MΩ or changed by more than 20%, the data were rejected for analysis. The current and voltage signals were recorded and digitized with a MultiClamp 700B amplifier and Digitdata 1440 controlled by Clampex 10 data acquisition software (Molecular Devices).

Quantification and statistical analysis

Student's t-tests were used to perform statistical comparisons within sexes between control and ELVS groups. Alpha levels of $p < 0.05$ were considered significant. All results are expressed as the mean ± SEM. Analyses and graphing were conducted in Clampfit 10.0, Easy Electrophysiology, Igor Pro 8 and SPSS 29.0 (IBM.)

Results

ELVS results in female-specific hyperactivity and impaired short-term memory

To determine how early life stress may affect affective, cognitive, and locomotive behaviors associated with LC function of male and female mice differently, we developed a two-hit stress model exposing mice to stress during early life (P6-16) and adolescence (P28-P42). We chose this model due to two-hit stress models providing more robust behavioral effects and the adolescent time point being particularly relevant to females' increase in stress-related psychiatric disorders. At 6-8 weeks of age, control and ELVS male and female mice were evaluated on a behavioral battery that included open field, y-maze, elevated zero maze, and marble burying tests. Female ELVS mice displayed an increase in distance moved when compared to female controls that persisted across the 30 minutes of testing **(Figure 1A&B)**. Female ELVS mice also entered the center of the open field arena more frequently **(Figure 1D)**, but for both sexes the time spent in the center was similar between control and ELVS mice **(Figure 1C)**. A similar effect was seen in elevated zero maze, with both ELVS males and females entering the center more frequently but the cumulative duration in the open arm were not significantly different **(Figure 1F&G)**. Female ELVS mice, but not male ELVS mice, also show impaired short-term with reduced correct sequential alternations when compared to female controls **(Figure 1E)**. In an assessment of repetitive behavior (18,19), in a standard 30 min marble burying assay, male ELVS mice buried significantly fewer marbles relative to controls, while performance of female mice from both groups was similar (**Figure 1H**). Overall, the ELVS stress paradigm impaired short-term memory and induced hyperactivity specifically in females, impacting behavioral domains of ADHD short-term after dual-hit early life and adolescent stress.

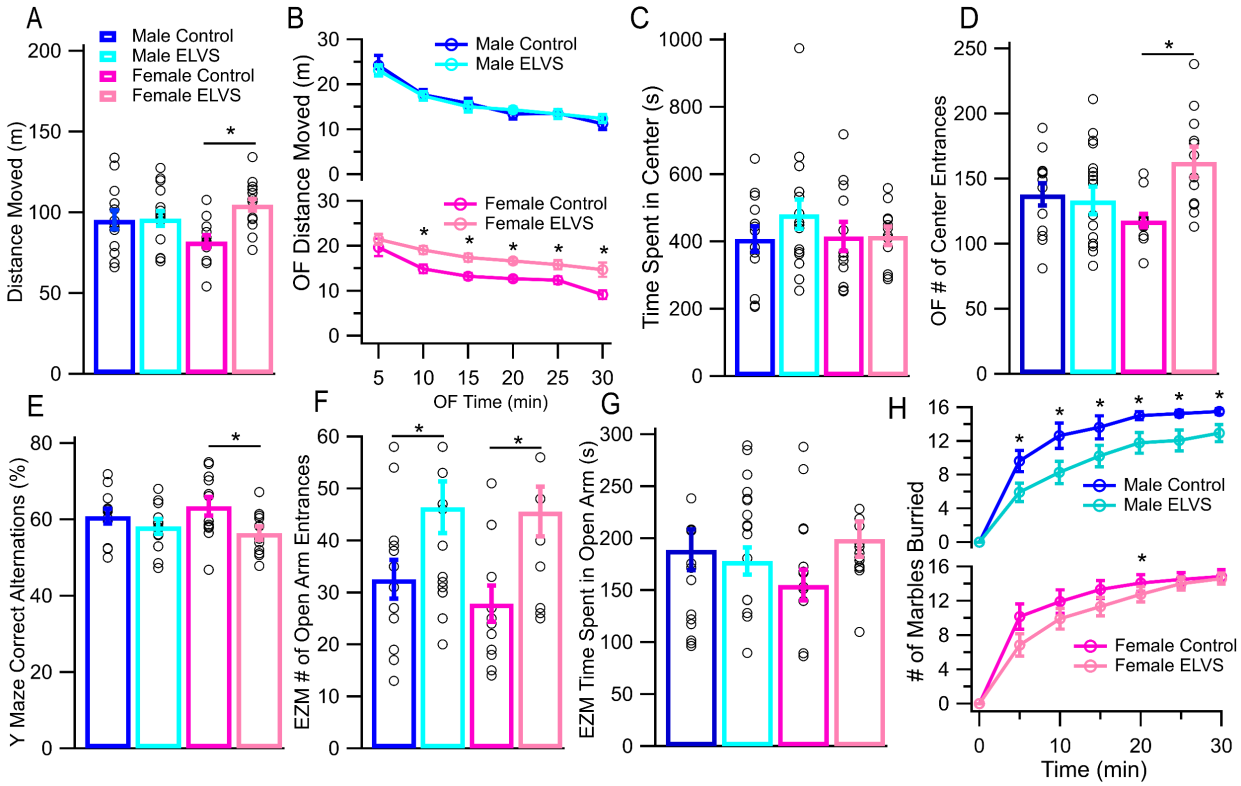

Figure 1. ELVS paradigm results in female-specific hyperactivity and short-term memory deficits along with a male specific decrease in marble burying. (A-D) Open field (OF) results (mean±SEM for each group) for total distance moved in meters (m) (A) distance moved every 5-minute interval of total time (B) time spent in the center of the OF in seconds (s) (C) and number of OF center entrances (D). (E) Y Maze results for correct alternation percentages (mean±SEM). (F&G) Elevated zero maze results (mean±SEM) for number of open arm entrances (F) and time spent in open arm (G). (H) Marble Burying test showing number of marbles buried at 5-minute interval increases ending at 30 minutes. N=12-16, 6 weeks old. *= $p<0.05$ t-test.

ELVS Female LC Neurons are less excitable and show increased action potential delay.

Given the sexual dimorphism of the behavioral phenotype produced by ELVS and the reported basal LC physiology, we performed current clamp recordings from putative LC noradrenergic neurons **(Figure 2A)** in *ex vivo* acute brain slices to determine whether ELVS

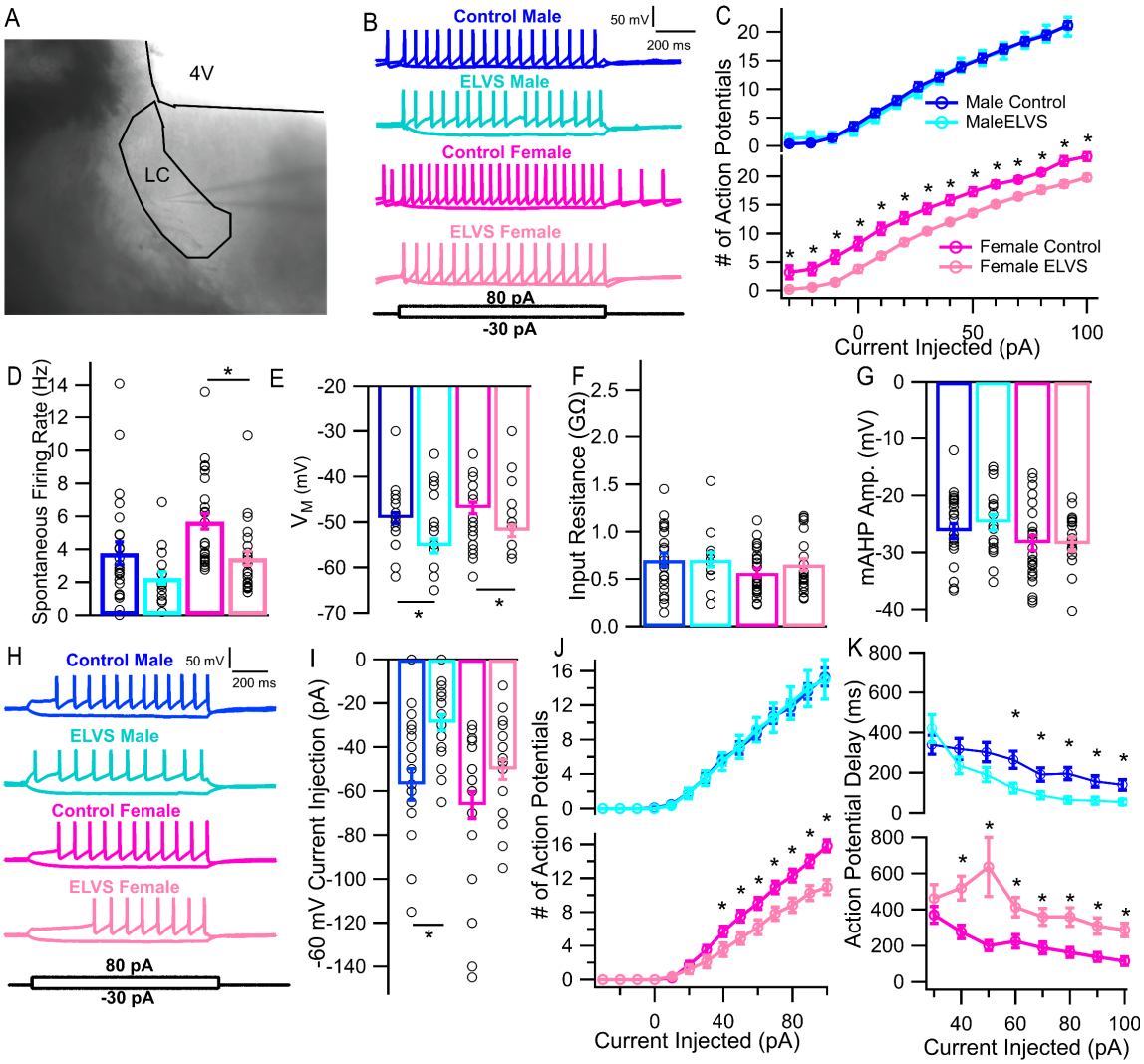

Figure 2. ELVS results in female-specific LC neuron evoked excitability reduction due to increased action potential delay. (A) Patch pipette on LC neuron at 10X. (B,C,H,J) Representative membrane potential recording (B, H) and mean±SEM action potential (AP) number (C, J) in response to current injection from LC neurons in all groups at natural resting potential (B,C) or initially held at -60mV (H,J). (D-G) Basal cellular characteristics of LC neurons. Firing rate at rest in Hertz (Hz) (D), resting membrane potential (V_m) in millivolts (mV) (E), input resistance in gigaohms (GΩ) (F), and maximum after hyperpolarization amplitude in mV (G). (I) Current in picoamps (pA) required to keep V_m at -60mV. (K) Time in milliseconds (ms) until first action potential due to current injection with cell held at -60 mV. N=2-3, n=21-28. 7-8 weeks. *= $p<0.05$ t-test.

injection-evoked excitability when compared to controls, a sex difference which was not observed between control and ELVS males **(Figure 2B&C)**. Noradrenergic LC neurons from female ELVS mice, but not males, also were found to have reduced spontaneous firing rates and hyperpolarized resting membrane potentials when compared to controls (**Figure 2D&E)**. However, there were no significant stress-related differences in input resistance or mAHP amplitudes **(Figure 2F&G)** in either sex.

To determine the degree to which the resting potential (Vm) difference between groups impacted excitability, an additional series of recordings was completed in the same cells with the initial resting potential adjusted to -60mV with current injection. With a Vm=-60 mV, most neurons were not spontaneously active **(Figure 2H)** and more negative current was required to hyperpolarize control male and female noradrenergic neurons relative to those form ELVS mice **(Figure 2I)**. Repeat injection of positive current injection, now from the same Vm of -60 mV was found to induce action potential firing only after a delay over the initial 100-400 ms of current injection **(Figure 2H)**. From an initial resting potential of -60mV in all neurons, ELVS females continued to show reduced evoked excitability **(Figure 2H, J)**, which appeared to be due to an increase in the delay time to the first action potential following current injection **(Figure 2J&K)**. Surprisingly, putative noradrenergic LC neurons form ELVS male mice had an opposing reduction in action potential delay **(Figure 2K)**, further highlighting a female-specific LC physiology change that could contribute to the observed sexually dimorphic LC-involved behavioral differences.

Male LC CRF_1's effect on action potential firing is calcium-dependent.

To understand how stress signaling may have differential impacts on noradrenergic neurons in the LC of male and female C57bl/6J mice, we wanted to whether CRF1 receptor activation produced sexually dimorphic effects on LC neuron excitability in naïve mice. We found that both male and female LC putative noradrenergic neurons increased spontaneous firing upon CRF bath application, which increased steadily over 15 min with peak CRF-induced increases in

excitability peaking at 10 min and females peaking at 15 min. Similar CRF-induced increases in current injection-evoked firing were observed in putative noradrenergic LC neurons in both males and females **(Figure 3C&D)**. Although it has previously been reported that female LC CRF_1 receptor is predominantly coupled G_s signaling, since CRF_1 can couple to G_s, G_q, and G_i pathways, we went on to determine the role of G_q signaling in CRF-induced increases in LC neuron excitability in both sexes. To assess the dependence of G_q-mediated signaling in the CRF-induced changes, a second series of recordings was performed with 10mM BAPTA, a Ca^{2+} chelating agent in the internal pipette solution to block G_q-Ca^{2+}-driven excitability changes **(Figure 3)**. In the presence of intracellular BAPTA, CRF no longer increased the spontaneous **(Figure 3A,B)** or current-evoked **(Figure 3C)** firing of male noradrenergic LC neurons. In contrast, the inclusion of intracellular BAPTA to remove cytosolic Ca^{2+} had no effect on CRF-induced changes in the spontaneous **(Figure 3A,B)** or evoked **(Figure 3D)** firing behavior of female noradrenergic LC neurons. Together, these data suggested that CRF_1 receptors were preferentially coupled to G_q signaling pathways in males and G_s signaling pathways in females, which may explain how stress signaling may lead to sexually dimorphic long term changes.

To determine the accuracy of our targeting putative noradrenergic LC neurons recorded in wildtype C57bl/6J mice, we repeated these experiments in transgenic mice expressing tdTomato driven by noradrenergic marker, dopamine β-hydroxylase, (Dbh-tdTomato mice). Baseline and CRF-induced changes in firing behavior of tdTomato+/noradrenergic LC neurons were similar to baseline and CRF-induced changes in C57bl/6J mice of both sexes **(Figure 4)**. Because the generation of Dbh-tdTomato mice relies on a knock-in of Cre in place of one copy of the *Dbh* gene, mechanistic studies were not performed in these mice due to potential unaccounted for compensatory changes that may occur in the absence of one copy of *Dbh*.

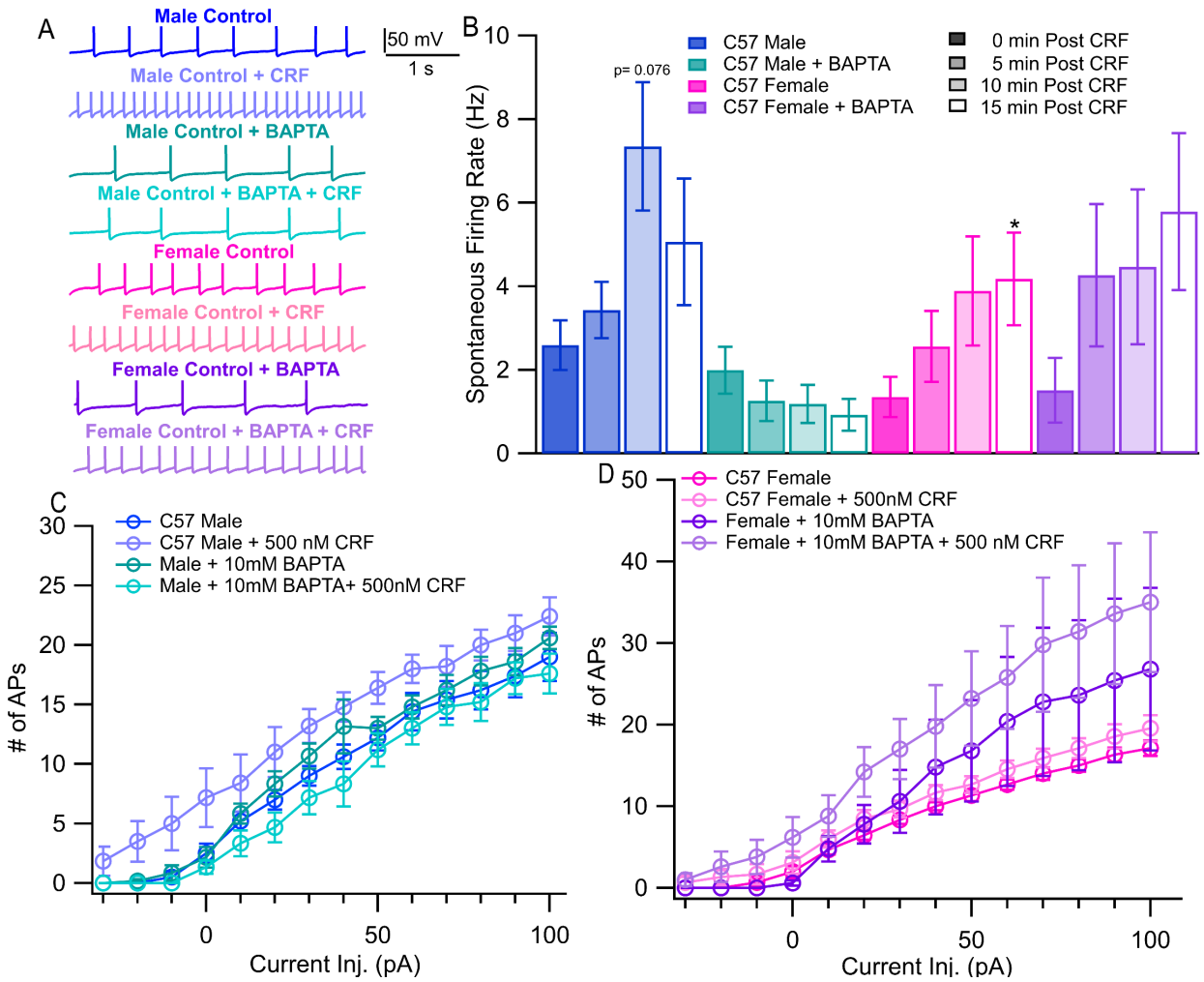

Figure 3. Sexually dimorphic LC neuron responses to CRF. (A) Representative traces of spontaneous firing LC neurons for male and female in ACSF, after 500nM CRF, and with or without intracellular BAPTA (10 mM) to chelate calcium. (B) Mean ± SEM spontaneous firing rate. (C&D) Mean ± SEM current injection-evoked action potential # for LC neurons in males (C) and females (D). N=2-4, n=9-11. 5-6 weeks old. *= $p<0.05$ t-test.

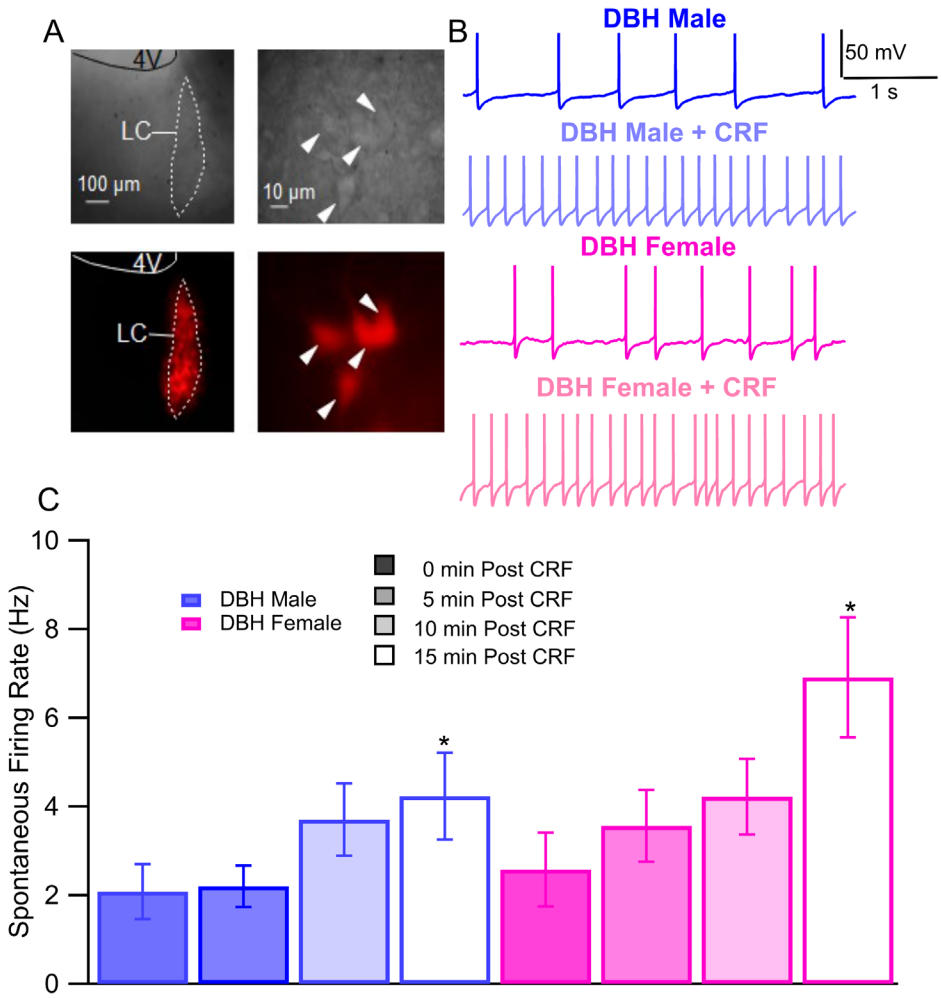

Figure 4. Similar LC neuron responses to CRF in DBH-tdTomato mice. (A) DIC (top) and fluorescent tdTomato (bottom) image of two LC neurons in DBH-tdTomato mice. (B) Representative traces of spontaneous firing LC neurons for male and female in ACSF, after 500nM CRF (C) Mean ± SEM spontaneous firing rate. N=2-4, n=9-11. 5-6 weeks old. *= $p<0.05$ t-test.

Discussion

Exposure to stress, especially at critical periods in early life, has clinically relevant impacts on behavior and risk for developing certain neuropsychiatric disorders that vary with sex. In our effort to develop an animal model to understand the underlying mechanisms involved early life stress-induced changes in cognitive and affective behavior, we found that a two-hit stress

exposure during P6-10 and during P28-42 reliably altered the locomotion, anxiety, and short-term memory of mice in early adulthood. Using these mice, we went on to identify a consistent hyperpolarization of the noradrenergic LC neuron resting potential of male and female mice exposed to stress and that, specifically in females, these neurons also display a prolongation of the delay to firing when animals had previously been exposed to stress. Based on these data, we hypothesize that stress leads to persistent increase in activity of constitutively active K+ channels that set the resting membrane potentials and activity of transient K+ channels that slow the rate of depolarization upon positive current injection/excitatory current in females after stress. Since these sex-specific changes likely stem from sexual dimorphism in stress hormone signaling in noradrenergic LC neurons, our effort to identify differences in CRF-driven activity changes in LC neurons indicate that CRF_1 receptors increase excitability of LC neurons in males via G_q-mediated mechanisms and in females likely by G_s-mediated mechanisms. Together, these data provide evidence that fundamental sex-differences in neuronal signaling predispose those neurons (noradrenergic LC) to developing different persistent long-term changes in response to appropriately-timed physiological and psychosocial stressors that may shape both conceptualization and possibly treatment of certain neurodevelopmental and psychiatric disorders.

Animal and human studies have shown that brain regions involved in stress, anxiety and cognition have sex differences that can be both structural and functional (20–22). The data presented here shed light on how the LC-NE system changes with multiple early life stress exposures depending on sex. After dual-hit early life stress, females had impaired LC spontaneous and evoked firing, which could result in a reduction of tonic and phasic LC firing *in vivo* leading to reduced overall signaling to systems involved in attention, arousal, memory, and locomotion (i.e. prefrontal cortex, hippocampus, ventral tegmental area, and lateral cerebellar nucleus). Further effort to determine exactly how these changes in LC-NE system excitability alters wide-spread noradrenergic signaling in vivo during specific tasks would provide support for

the importance of this physiological change and suggest the LC-NE system to be a female specific target of interest for stress-related disorders, particularly with onset after adolescence.

The sexually dimorphic mechanism for LC physiology changes after exposure to the ELVS stress paradigm is still unclear. Our results in non-stressed mice validate that there are basal sex differences in LC CRF_1 coupling and suggest male CRF_1 require calcium to elicit a response, indicating a predominant G_q coupling. Female CRF_1 in the LC has been shown to be predominantly G_s coupled, and males have increased β-Arestin signaling internalizing CRF_1 after acute stress (28). These fundamental sex differences in CRF_1 signaling and trafficking in the LC may underlie sexually dimorphic changes in LC physiology after stress. Future studies should explore if females exposed to prolonged stress have reduced CRF_1 internalization in the LC compared to males and experience chronic PKA signaling cascade activation through G_s activation that ultimately reduce LC neuron excitability when compared to males. The calcium mediated G_q coupling predominance could also be protective in males due to its transient action based on calcium stores versus a prolonged upregulation of a signaling cascade, and should be explored further. It has also been reported that acute stress exposure elicits persistent LC physiology changes only if done at adolescence and not adulthood (24). The mechanism of increased LC sensitivity to stress during adolescence should be explored further. Overall, linking the sex differences in the basal state of the LC neuron's stress response to what is observed in our model after dual-hit early life stress will reveal the root of the LC physiology changes to determine if they can be reversed.

PTSD treatments have been shown to be highly variable in providing successful outcomes, with little data displaying long-term efficacy of current treatment methods. The nonresponse and dropout rates of PTSD efficacy studies are often as high as 50%, with 60-72% of patients across recent PTSD treatment clinical trials continuing to display clinically significant life impairments (25,26). Considering sex and the timing of stress exposure may be biological variables influencing patient outcomes. The behavioral results suggest females are more

sensitive to memory deficits and hyperactivity as a result of dual-hit early life stress. The adolescent time point may be particularly relevant to this change due to major sex differences in development at this time. However, the specific role of sex hormones in development and neurophysiological changes in response to stress is unclear. Estrogen receptor α (ERα) and β (ERβ) are differentially expressed in the amygdala, prefrontal cortex, and hippocampus responsible for stress, anxiety, and cognitive behaviors respectively (27). ERβ has been associated with non-reproductive behaviors, with its activation improving memory and reducing anxious behaviors in females (28,29). In contrast, testosterone signaling has been suggested to be a factor supporting resilience to stress-induced changes, which is in line the behavioral data presented here (30,31).

This study may implicate the adrenergic system, particularly the LC in the brain, as a region to explore biomarkers of dysfunction in female patients with a history of stress in early life. There are many drugs that target this brain area, particularly second-line therapies for attention deficit hyperactivity disorder (ADHD), which may prove to be effective in managing cognitive impairments in this patient group with PTSD. These data provide a foundation for further research into neuro-stress regulation differences in males and females, relevant to many stress-related disorders from PTSD to substance abuse disorder that have sex differences in their prevalence ratios (17).

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Acknowledgements

This work was supported by departmental start-up funds (BDR).