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Long-Term Impact of Early Life Stress on Serotonin Connectivity

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ABSTRACT

BACKGROUND: Chronic childhood stress is a prominent risk factor for developing affective disorders, yet mechanisms underlying this association remain unclear. Maintenance of optimal serotonin (5-HT) levels during early postnatal development is critical for the maturation of brain circuits. Understanding the long-lasting effects of early life stress (ELS) on serotonin-modulated brain connectivity is crucial to develop treatments for affective disorders arising from childhood stress.

METHODS: Using a mouse model of chronic developmental stress, we determined the long-lasting consequences of ELS on 5-HT circuits and behavior in females and males. Using FosTRAP mice, we cross-correlated regional c-Fos density to determine brain-wide functional connectivity of the raphe nucleus. We next performed in vivo fiber photometry to establish ELS-induced deficits in 5-HT dynamics and optogenetics to stimulate 5-HT release to improve behavior.

RESULTS: Adult female and male mice exposed to ELS showed heightened anxiety-like behavior. ELS further enhanced susceptibility to acute stress by disrupting the brain-wide functional connectivity of the raphe nucleus and the activity of 5-HT neuron population, in conjunction with increased orbitofrontal cortex (OFC) activity and disrupted 5-HT release in medial OFC. Optogenetic stimulation of 5-HT terminals in the medial OFC elicited an anxiolytic effect in ELS mice in a sex-dependent manner.

CONCLUSIONS: These findings suggest a significant disruption in 5-HT–modulated brain connectivity in response to ELS, with implications for sex-dependent vulnerability. The anxiolytic effect of the raphe–medial OFC circuit stimulation has potential implications for developing targeted stimulation-based treatments for affective disorders that arise from early life adversities.

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Exposure to early life stress (ELS) is associated with alterations in brain function and emotional behavior and can contribute to the development of psychiatric disorders in adulthood (1–3). Research over the past 3 decades has established the long-lasting effects of ELS on the risk and course of mood and anxiety disorders (4). Early traumatic experiences correlate with aberrant hypothalamic-pituitary-adrenal axis reactivity (5), altered brain activity (6,7), and impaired social and emotional behaviors (8), with an overall increased risk for lifetime affective disorders (9–11). Brain regions with extended postnatal development including higher-order cortical areas, such as the dorsolateral prefrontal cortex (PFC) and orbitofrontal cortex (OFC), are suggested to have increased vulnerability to the negative effects of ELS (12,13).

The neurotransmitter serotonin (5-HT) is widely distributed across the central nervous system and is critical for social and emotional regulation. During early development, 5-HT regulates cell survival, growth, differentiation, and maturation of neural circuits (14,15). Peak 5-HT levels occur during the first 2 years of life in humans (16) and the first postnatal week in rodents (17). Manipulations that alter the serotonin system during these periods, such as stress or maternal absence (18–21), are associated with chronic behavioral deficits (22–25). 5-HT neurons originate from the 9 distinct nuclei clustered in the brainstem and project widely throughout the brain providing widespread modulation of the activity of many neural networks (26,27). The dorsal raphe nucleus (DRN) contains the majority of 5-HT–producing neurons, which are highly heterogeneous in terms of physiological function and gene expression profiles (26,28,29). There is considerable overlap in the response of DRN 5-HT neurons to differentially salient stimuli; however, distinct projection subpopulations can show functional bias based on their downstream connectivity. While a mixed population of DRN 5-HT neurons are activated by reward (29–32), those projecting to cortical and subcortical regions encode opposing responses to aversive stimuli (30). Overall, DRN 5-HT neurons play a critical role in environment-specific behavioral regulation when faced with positive stimuli or threat (33).

Early postnatal stress can disrupt the maturation and activity of the 5-HT circuits, leading to persistent negative effects

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on emotional behavior and threat response [reviewed in (22,34,35)]. It is imperative to develop targeted treatments to overcome ELS-induced behavioral dysregulation. Yet, we do not fully understand how distinct 5-HT projections involved in emotionally salient behavior are impacted by ELS. Here, we established a chronic ELS model based on the limited bedding and nesting (LBN) paradigm (36) in female and male mice. When ELS offspring reached adulthood, we examined threat-induced alterations in raphe nucleus (RN) functional connectivity, 5-HT neuron activity, and 5-HT release. We found that ELS disrupts functional connectivity of the RN and perturbs the response of 5-HT neurons to threat imposed by footshock. This is accompanied by increased activity in the OFC but not central amygdala (CeA), two regions that receive dense 5-HT innervation and encode emotionally salient stimuli. We observed blunted 5-HT release in the medial OFC (mOFC) of mice subjected to ELS in the face of challenge, along with disrupted 5-HT signaling. Optogenetic stimulation of 5-HT projections in the mOFC improved anxiety-like behavior observed in male ELS mice, suggesting a sex-dependent anxiolytic effect. Ultimately, these findings have important implications for stimulation-based treatment approaches.

METHODS AND MATERIALS

See Supplemental Methods in Supplement 1 for extended details.

Animals

Female and male Fos[2A-iCreER] (TRAP2) (JAX 030323) mice crossed with Ai14 reporter line (JAX 007914) and ePet1-cre (JAX 012712) mice crossed with Ai148 reporter line (JAX 030328) on C57BL/6 background were used for all experiments (all mice from The Jackson Laboratory).

Limited Bedding and Nesting

LBN was performed as previously described (36) on postnatal days (PNDs) 2 to 10 with modifications in the protocol to enhance pup survival. Maternal behavior was recorded during light and dark cycles.

Network Connectivity and FASTMAP Analysis

For functional connectivity analyses, c-Fos immunoreactive density was assessed across 64 regions (Table S1 in Supplement 1) using NeuroInfo software (MBF Bioscience). For targeted c-Fos density analyses, FASTMAP (37) was used for registration of the OFC and CeA.

Stereotactic Viral Delivery and Fiber Implantation

For fiber photometry experiments, 400 nL AAV2/9-CAG-iSeroSnFR-NLG (Canadian Neurophotonics Platform) was infused into the mOFC (anteroposterior 2.34, mediolateral 0.3, and dorsoventral 2.5 mm from bregma), followed by optic fiber implantation. For ePet1::Ai148 mice, the optic fiber was implanted above the DRN (30° angle; anteroposterior −6.27, mediolateral 0, and dorsoventral −4.04 from bregma). For optogenetic experiments, 800 nL AAV2/9-EF1a-DIO-mCherry or AAV2/9-EF1a-DIO-hChR2(H134R)-mCherry was infused into the DRN. Optic cannulae were implanted above the mOFC bilaterally (21° angle; anteroposterior 2.34, mediolateral ±1.25, dorsoventral −2.6 from bregma).

In Vivo Fiber Photometry

Ca^{2+} signal of the 5-HT neuron population was recorded in ePet1::Ai148 mice to measure 5-HT neuron activity in response to footshocks (0.5 mA, 10 times, 2 seconds each). 5-HT release in mOFC was recorded in mice infused with the iSeroSnFR 5-HT sensor during the open field test and tail suspension test (TST). Data were extracted and analyzed using a custom-written script in MATLAB (version 2020a; The MathWorks, Inc.).

Statistical Analysis

Data were analyzed using two-way analysis of variance or unpaired *t* test (GraphPad Prism 9; GraphPad Software). Following significant interactions in two-way analysis of variance, post hoc analysis was conducted using Tukey's multiple comparisons tests. The complete statistical output is included in Table S2 in Supplement 2.

RESULTS

LBN Differentially Affects Dam Behavior in Light and Dark Cycles

To model ELS, we used a modified LBN paradigm (PNDs 2–10) (Figure 1A). Consistent with previous studies (36,38,39), we found alterations in maternal behavior in LBN dams compared with controls. Maternal behavior analysis showed that control and LBN dams spent comparable time with pups during daytime (Figure 1B). During nighttime, control dams spent significantly less time with pups compared with LBN dams, predominantly on PND 6 and PND 9 (Figure 1C). During both day and night, LBN dams exhibited more nest entries and exits compared with controls (Figure 1D, E). Overall, LBN dams showed the largest differences in maternal care during the dark cycle, with greater time spent with pups accompanied by increased frequency of nest entries and exits. These may indicate attempted compensatory care to overcome the impoverished housing conditions.

Female and Male Offspring Exposed to LBN Show Anxiety-Like Behavior in Adulthood

To determine long-lasting behavioral changes in offspring reared under ELS, we performed open field test, 3-chamber social interaction test, and TST when pups reached adulthood (>PND 60) (Figure 2A). Compared with controls, ELS female and male mice spent greater time in the outer zone and reduced time in the central zone of the open field, indicating greater anxiety-like behavior (Figure 2B–D), consistent with earlier studies [reviewed in (36)]. Distance traveled in the open field differed between control and ELS mice, with significant sex-dependent effects (Figure S1A in Supplement 1).

Next, we performed the 3-chamber social interaction test to assess sociability. The time spent interacting with sex- and age-matched stranger conspecifics was comparable between ELS and control mice (Figure 2E). Female and male ELS mice spent greater time in the non–social interaction zone (Figure 2F), but were comparable to controls with respect to

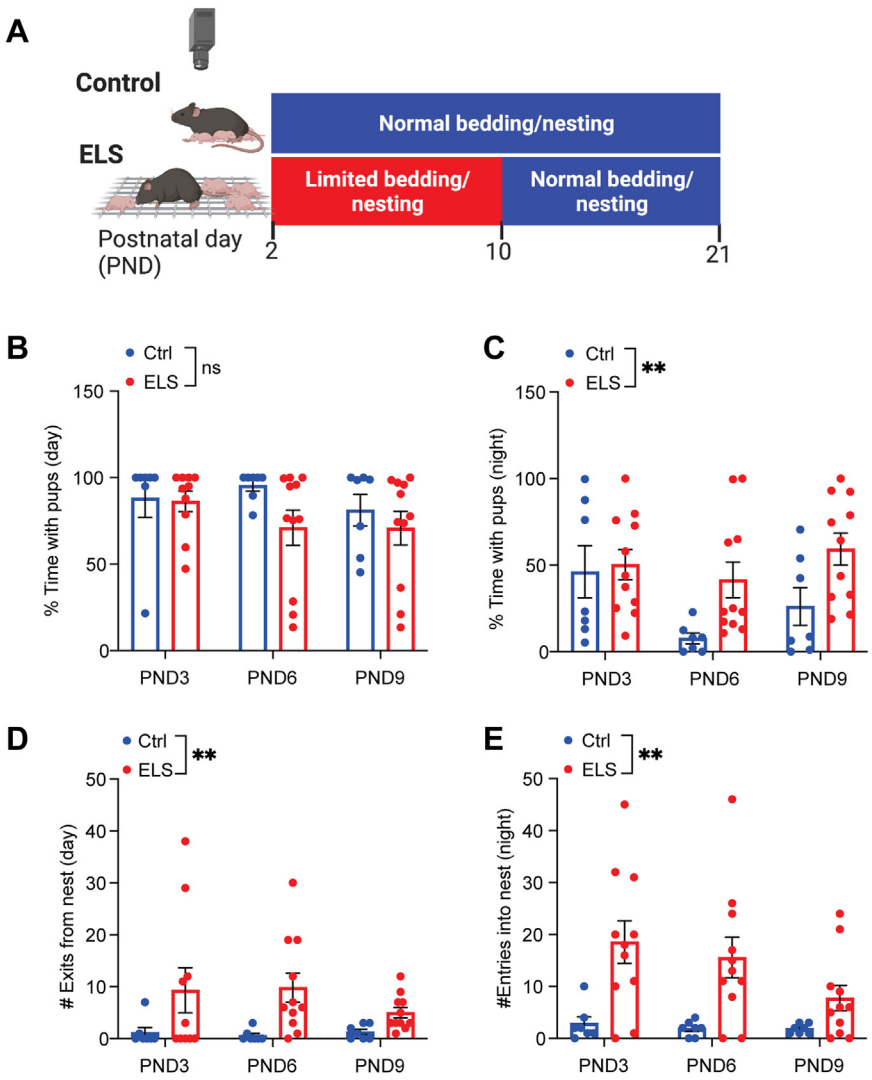

Figure 1. Maternal behavior during light and dark cycle. **(A)** Experimental paradigm showing the implementation of Ctrl and LBN conditions. Dams and pups were exposed to LBN on PNDs 2–10. **(B)** Time LBN dams (n = 11) spent with pups on PNDs 3, 6, and 9 during the light cycle was comparable to Ctrl dams (n = 7) (two-way ANOVA; effect of group: $F_{1,48}$ = 2.51, p = .12, effect of time: $F_{2,48}$ = 0.86, p = .43). **(C)** LBN dams spent significantly greater time with pups across PNDs 3, 6, and 9 during the dark cycle compared with Ctrl dams (two-way ANOVA; effect of group: $F_{1,48}$ = 7.79, p = .007, effect of time: $F_{2,48}$ = 2.84, p = .07). **(D)** LBN dams exhibited significantly higher frequency of nest exits on PNDs 3, 6, and 9 during the light cycle compared with Ctrl dams (two-way ANOVA; effect of group: $F_{1,48}$ = 10.02, p = .003, effect of time: $F_{2,48}$ = 0.35, p = .71). **(E)** During the dark cycle, LBN dams exhibited significantly higher frequency of nest entries compared with Ctrl dams (two-way ANOVA; effect of group: $F_{1,48}$ = 19.95, $p < .001$, effect of time: $F_{2,48}$ = 1.74, p = .19). Data represent mean ± SEM; $^{**}p < .01$. ANOVA, analysis of variance; Ctrl, control; ELS, early life stress; LBN, limited bedding and nesting; ns, nonsignificant; PND, postnatal day.

sociability (Figure S1B in Supplement 1). Further analysis revealed that both female and male ELS mice entered the social and non–social interaction zones at a greater frequency (Figure S1C, D), but spent significantly less time per visit in each zone compared with controls (Figure S1E, F), which can be due to increased anxiety rather than deficits in sociability.

To investigate the effect of a stressful environment on coping behaviors in control and ELS mice, we conducted the TST, which acts as an inescapable stressor in a high-threat environment (33). We quantified the time mice engaged in active (struggling behavior) and passive (immobility) coping behaviors (40). Female ELS mice spent significantly greater time engaged in active coping behavior compared with control females and ELS males. We did not find a difference in TST between control males and ELS males (Figure 2G).

Together, these data suggested that female and male offspring reared under ELS show striking increases in anxiety-like behavior. Female ELS mice additionally show greater inescapable stress–induced active coping behavior during adulthood.

ELS Disrupts RN Functional Connectivity

To understand how mice exposed to ELS respond to threat, we utilized footshock, a commonly used acute stress paradigm. The use of a footshock paradigm additionally helped us to activate and tag cells that are responsive to stress in both control and ELS mice. To do this, we used FosTRAP2::tdTomato mice, in which cells activated during a discrete temporal window can be permanently tagged with the fluorescent reporter tdTomato (41). We correlated footshock-induced regional c-Fos–immunoreactive densities across mice within each group and generated activity matrices (Figure 3A). Using the RN as a seed region, we assessed its functional connectivity with 64 brain regions

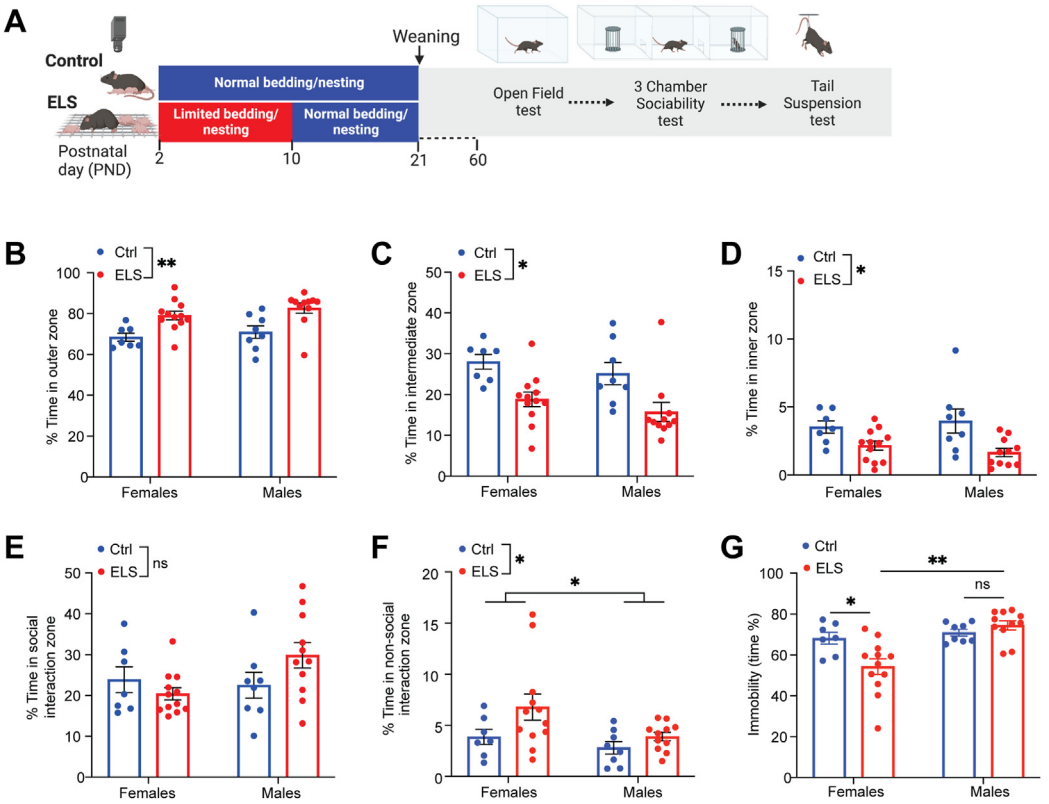

Figure 2. Long-term impact of ELS on offspring behavior. **(A)** Experimental paradigm showing behavioral tests performed after offspring reared under Ctrl and limited bedding and nesting conditions reached adulthood (>postnatal day 60). **(B–D)** Female (n = 12) and male (n = 11) ELS mice spent a significantly greater amount of time in the outer zone (two-way ANOVA; effect of group: $F_{1,34}$ = 19.69, $p < .001$, effect of sex: $F_{1,34}$ = 1.48, p = .23) **(B)** and significantly less time in the intermediate (two-way ANOVA; effect of group: $F_{1,34}$ = 16.84, $p < .001$, effect of sex: $F_{1,34}$ = 1.77, p = .19) **(C)** and inner (two-way ANOVA; effect of group: $F_{1,34}$ = 13.52, $p < .001$, effect of sex: $F_{1,34}$ = 0.006, p = .94) **(D)** zones of the open field, indicating greater anxiety-like behavior compared with female (n = 7) and male (n = 8) Ctrl mice. **(E)** Time spent interacting with a stranger mouse (social interaction zone) was comparable between female and male Ctrl and ELS mice (two-way ANOVA; effect of group: $F_{1,34}$ = 0.49, p = .49, effect of sex: $F_{1,34}$ = 2.15, p = .15). **(F)** Female and male ELS mice spent significantly greater time interacting with an empty cup (non–social interaction zone) compared with Ctrl mice (two-way ANOVA; effect of group: $F_{1,34}$ = 4.47, p = .04, effect of sex: $F_{1,34}$ = 4.35, p = .04). **(G)** Female ELS mice showed reduced passive coping behavior (immobility) during the tail suspension test compared with female Ctrl mice and male ELS mice (two-way ANOVA; group × sex interaction: $F_{1,34}$ = 7.78, p = .009, Tukey's multiple comparisons test: female Ctrl vs. ELS p = .02; female ELS vs. male ELS $p < .001$, male Ctrl vs. ELS p = .84). Data represent mean ± SEM; $^{*}p < .05$, $^{**}p < .01$. ANOVA, analysis of variance; Ctrl, control; ELS, early life stress; ns, nonsignificant.

(Figure 3B). The number of brain regions showing anticorrelated activity with the RN was greater in ELS mice compared with controls, with the most striking differences present in ELS males (Figure 3C, D; Table S1 in Supplement 1). This suggested that ELS led to an increase in anti-correlated functional connectivity of the RN, signifying a loss of coordinated activity between the RN and numerous brain regions. We plotted the distribution of Pearson's correlation coefficients for all possible correlations in each network. This revealed significant differences between control and ELS males, control and ELS females, and ELS females and males. We did not see a difference in the distribution of R values between female and male control groups (Figure 3E).

The functional connectome of the RN in ELS mice revealed a higher number of anticorrelated connections, with males showing a greater disruption in connectivity compared with females. This suggests that offspring reared under ELS display reduced synchronization between the RN and other brain regions in adulthood.

ELS Disrupts Serotonin Neuron Activity in Response to an Aversive Stimulus

The RN contains a heterogeneous neural network composed predominantly of 5-HT neurons that are crucial for mediating threat adaptive behaviors (33). To examine threat-induced differences, we recorded the population activity of DRN 5-HT neurons in response to footshock (0.5 mA, 10 times, 2-second duration each) in the ePet1-Cre::Ai148 mouse line that expresses GCaMP6f specifically in 5-HT neurons (Figure 4A–C; Figure S2A–C in Supplement 1). The averaged Ca^{2+} signal from the 5-HT neuron population yielded a biphasic response to footshock with an initial increase in activity followed by decreased activity in all mice (Figure 4D, E). We analyzed the footshock-induced increase (peak) and

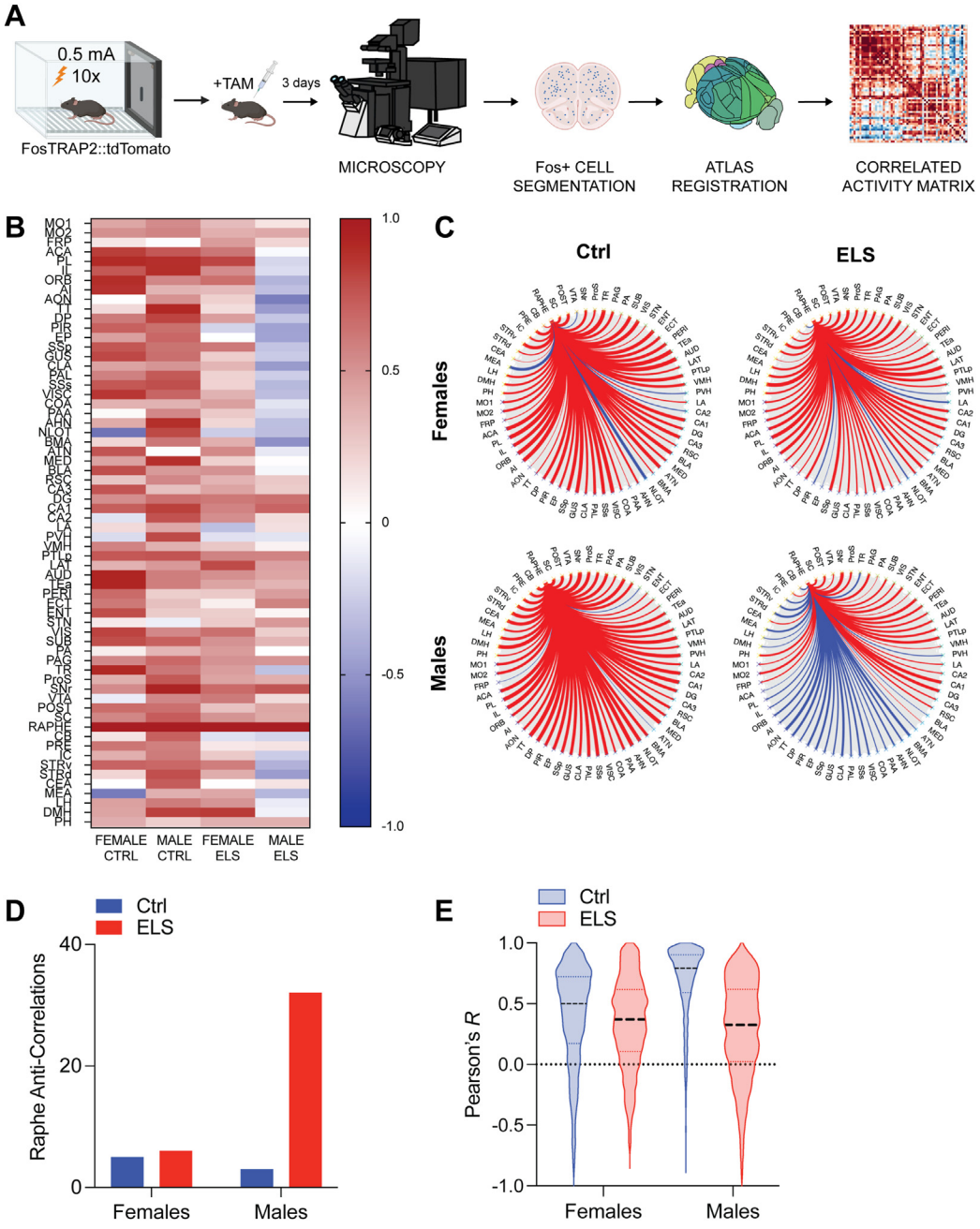

Figure 3. ELS-induced disruption in raphe functional connectivity. **(A)** Pipeline for assessing functional connectivity underlying acute footshock stress in female Ctrl (n = 7), male Ctrl (n = 7), female ELS (n = 11), and male ELS (n = 10) mice. **(B)** Functional connectivity of the raphe nucleus based on the regional c-Fos activity was assessed by isolating the column in the correlated activity matrix corresponding to this region for female and male Ctrl and ELS mice. **(C)** ELS led to an increase in anticorrelated connectivity in the raphe nucleus, which was more prominent in males. Blue lines depict negative correlations, and red lines depict positive correlations. Line weight is indicative of the magnitude of the Pearson R value. **(D)** Number of brain regions showing anticorrelated activity (negative correlations) with the raphe nucleus in female and male Ctrl and ELS mice. **(E)** Distribution of Pearson's correlation coefficients showed significantly altered correlation between Ctrl and ELS mice, with male ELS mice showing the most striking difference from Ctrl mice (Kolmogorov-Smirnov tests: female Ctrl vs. female ELS, $p = .0039$; male Ctrl vs. male ELS, $p < .0001$; female Ctrl vs. male Ctrl, $p = .059$; female ELS vs. male ELS, $p < .0001$). See Table S1 in Supplement 1 for the list of names for abbreviated brain regions. Ctrl, control; ELS, early life stress.

depression (trough) in the amplitude of the 5-HT Ca^{2+} signal between each group. In females, we found only a significant main effect of time (Figure 4F). However, in males, we found a significant main effect of both group (control, ELS) and time (Figure 4G). Footshock-induced peak 5-HT Ca^{2+} signal amplitude did not differ between the groups (Figure 4H). The

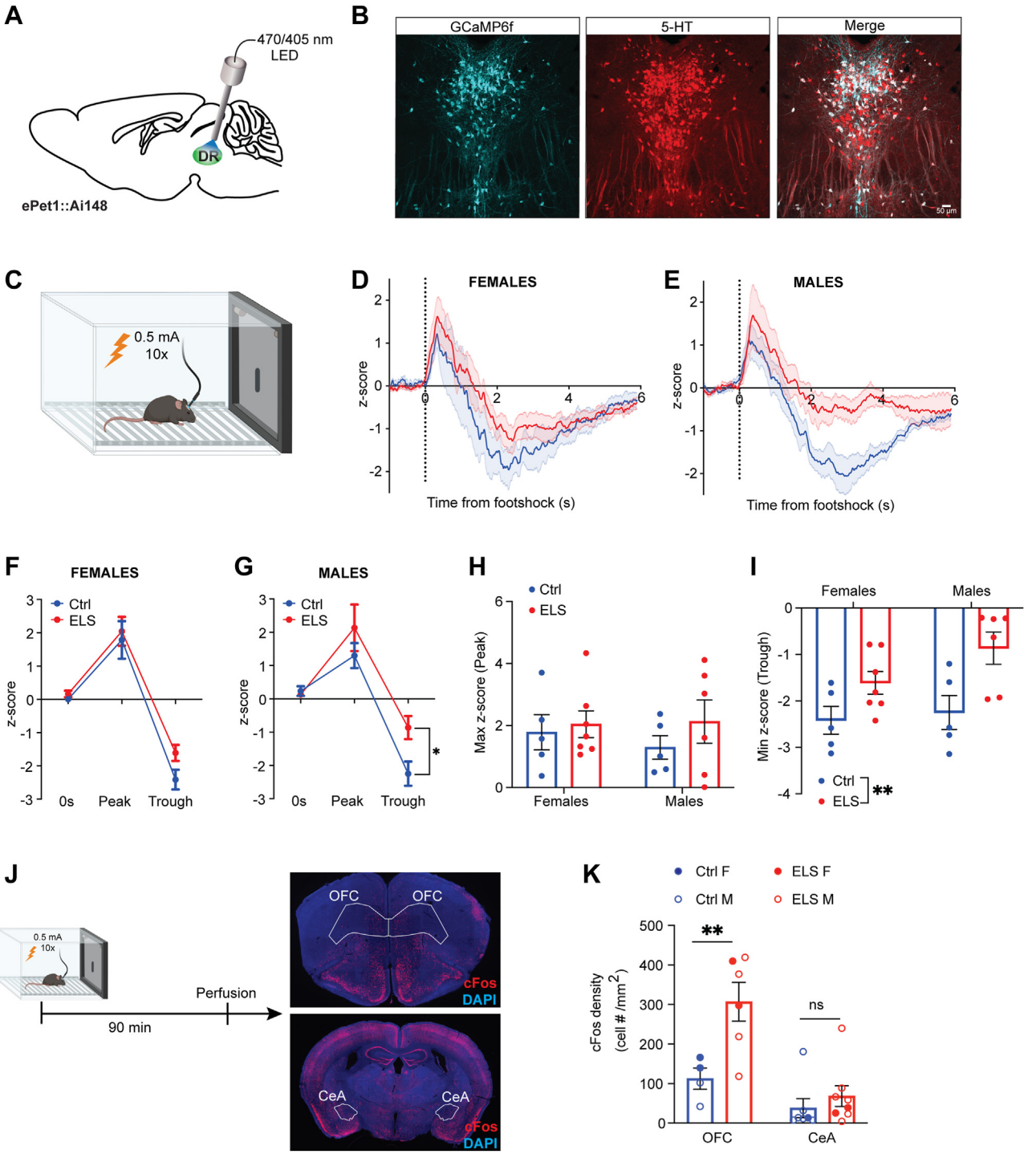

Figure 4. ELS alters footshock-induced activity of DR 5-HT neurons and postsynaptic regions. **(A)** Schematic showing implantation of an optical fiber in the DR of ePet1::Ai148 mice, allowing in vivo Ca^{2+} imaging in 5-HT neurons. **(B)** Representative images showing GCaMP6f (cyan) and 5-HT (red) colocalization in the DR of ePet1::Ai148 mouse. **(C)** Footshock paradigm (10 footshocks, 0.5 mA, each 2 seconds, presented at 30-second intervals). **(D–G)** Averaged Ca^{2+} signal changes in 5-HT neurons in female (Ctrl: $n = 5$; ELS: $n = 7$) **(D)** and male (Ctrl: $n = 5$; ELS: $n = 6$) **(E)** mice in response to acute footshock. The activity (z score) of 5-HT neuron population in response to footshock. The baseline at footshock onset (time = 0 seconds), the maximum response during peak (time = 0–1 second), and the minimum response during trough (time = 2–4 seconds) were plotted for female **(F)** and male **(G)** Ctrl and ELS mice (two-way ANOVA; females, effect of group: $F_{1,30} = 2.3$, $p = .14$, effect of time: $F_{2,30} = 71.32$, $p < .001$; males, effect of group: $F_{1,27} = 4.51$, $p = .04$, effect of time: $F_{2,27} = 32.48$, $p < .001$). **(H)** Footshock-induced maximum 5-HT neuron activity (peak) is comparable between female and male Ctrl and ELS mice (two-way ANOVA; effect of group: $F_{1,19} = 1.01$, $p = .33$, effect of sex: $F_{1,19} = 0.14$, $p = .72$). **(I)** Footshock-induced minimum 5-HT neuron activity (trough) is significantly reduced in female

magnitude of footshock-induced depression in 5-HT Ca^{2+} signal was significantly reduced in female and male ELS mice compared with controls (Figure 4I).

Footshock-induced biphasic changes in 5-HT neuron activity may be attributed to the heterogeneity of this population based on connectivity. Previous work demonstrated that DRN 5-HT neurons projecting to the OFC and CeA exhibit contrasting changes in activity in response to aversive stimuli (30). To determine whether acute footshock modulates the activity of these projection regions differentially in control and ELS mice, we perfused a group of mice 90 minutes after footshock and immunolabeled the brains for c-Fos (Figure 4J). FASTMAP analysis (37) revealed that ELS mice have greater c-Fos–immunoreactive cells in the OFC, while the c-Fos density in the CeA was comparable between control and ELS mice (Figure 4K).

Altogether, females and males subjected to ELS showed reduced footshock-induced depression in 5-HT Ca^{2+} signal, suggesting a disruption in 5-HT response to acute stress. Footshock-induced activity revealed regional differences, with greater c-Fos immunoreactivity in the OFC of ELS mice.

ELS-Induced Disruption in 5-HT Release in Avoidable and Unavoidable Threat Environments

To determine whether activity-dependent alterations in the OFC of ELS mice could be mediated in part by stress-induced deficits in 5-HT modulation, we investigated 5-HT release during threat-inducing environments (33). We injected the mOFC with AAV2/9-CAG-iSeroSnFR-NLG (42) expressing the 5-HT sensor in C57BL/6 mice and implanted the optical fiber above this region (Figure 5A, B; Figure S3A, B in Supplement 1). Four weeks after viral infusion, we performed fiber photometry while mice investigated an avoidable threat environment (open field) followed by exposure to an unavoidable threat environment (TST) (Figure 5C).

Mice tend to perceive the inner zones of the open field as more aversive than the peripheral zones, likely due to the higher level of uncertainty. To investigate how 5-HT release is modulated during transition from a lower to higher risk environment, we quantified the iSeroSnFR signal as mice moved from the outer zone to the central zone (Figure 5D; Figure S3C in Supplement 1). The area under the curve of the iSeroSnFR signal in control and ELS mice was greater while mice were in the central zone, suggesting greater 5-HT release as mice transitioned to a more aversive environment (Figure 5E; Figure S4A–D in Supplement 1). Although area under the curve was not significantly different between the groups, the mean amplitude of the iSeroSnFR signal before and after entry into the central zone revealed a significant reduction in mOFC 5-HT release in ELS mice compared with controls (Figure 5F; Figure S4A–D).

The TST exposes mice to an inescapable stressor, eliciting passive and active coping behaviors in response. Building on our observations of enhanced mobility in ELS mice during the TST and the established link between chronic stress and altered coping strategies, we quantified the mOFC iSeroSnFR signal 2 seconds before and after the onset of immobility in mice (Figure 5G). Compared with controls, ELS mice exhibited a decrease in both area under the curve and mean amplitude of the iSeroSnFR signal in the TST (Figure 5H, I; Figure S4E–H in Supplement 1), with an overall increase in amplitude following immobility onset in both groups (Figure 5I; Figure S4E–H). Our results indicate that mOFC 5-HT release is greater during the transition to passive coping behavior in the TST; however, this release is significantly lower in ELS mice compared with controls, regardless of the coping strategy employed.

To investigate the physiological response of mOFC pyramidal neurons to 5-HT, we performed whole-cell patch-clamp electrophysiology in cortical slices obtained from control and ELS mice (Figure 5J). The amplitude of the outward 5-HT current was significantly smaller in mOFC neurons of ELS mice compared to that of controls (Figure 5K, L), while the majority of passive membrane characteristics did not differ (Figure S5A–D in Supplement 1). The spike threshold was significantly more depolarized in mOFC neurons of ELS mice, without changes in spike amplitude or intrinsic excitability (Figure S5E–H).

Our data showed that ELS is associated with a significant reduction in mOFC 5-HT release during risky and high-threat conditions. Additionally, we observed a reduced 5-HT inhibitory response in mOFC pyramidal neurons of ELS mice, indicating a disruption in the 5-HT-induced modulation of mOFC activity.

Optogenetic Stimulation of mOFC 5-HT Terminals Elicits an Anxiolytic Effect in Male ELS Mice

To investigate whether stimulation of mOFC 5-HT terminals is sufficient to improve ELS-induced deficits in behavior, we infused a Cre-dependent AAV (adeno-associated virus) encoding hChR2-mCherry (control: mCherry) into the DR of ePet1-Cre mice and implanted the optical fibers bilaterally over the mOFC ($DR^{5\text{-}HT} \rightarrow$mOFC) (Figure 6A). This led to robust expression of the virus in the DR 5-HT neurons and their processes in the mOFC (Figure 6B, C). Using slice electrophysiology, we verified that mOFC 5-HT terminal stimulation with blue light resulted in the suppression of action potentials in pyramidal neurons, which was abolished by application of the 5-HT_{1A} receptor antagonist WAY-100635 (Figure 6D). Five weeks after surgeries, we performed the open field test followed by the TST (Figure 6E). $DR^{5\text{-}HT} \rightarrow$mOFC optogenetic stimulation decreased the time spent in the outer zone and increased the time in the central zone of the open field in male

and male ELS mice compared with controls (two-way ANOVA; effect of group: $F_{1,19} = 12.14$, $p = .002$, effect of sex: $F_{1,19} = 2.11$, $p = .16$). **(J)** Experimental paradigm showing representative images of c-Fos staining in mice perfused 90 minutes after footshock. **(K)** ELS mice show increased activity in the OFC indicated by significantly greater number of c-Fos–positive cells induced by footshock compared with Ctrl mice. The activity in CeA is comparable between Ctrl and ELS mice (two-way ANOVA, group × brain region: $F_{1,21} = 5.77$, $p = .02$; Tukey's multiple comparisons test, OFC Ctrl vs. ELS: $p = .008$, CeA Ctrl vs. ELS: $p = .89$). Data represent mean ± SEM; $^{*}p < .05$, $^{**}p < .01$. ANOVA, analysis of variance; CeA, central amygdala; Ctrl, control; DR, dorsal raphe; ELS, early life stress; F, female; LED, light-emitting diode; M, male; Max, minimum; Min, minimum; ns, not significant; OFC, orbitofrontal cortex.

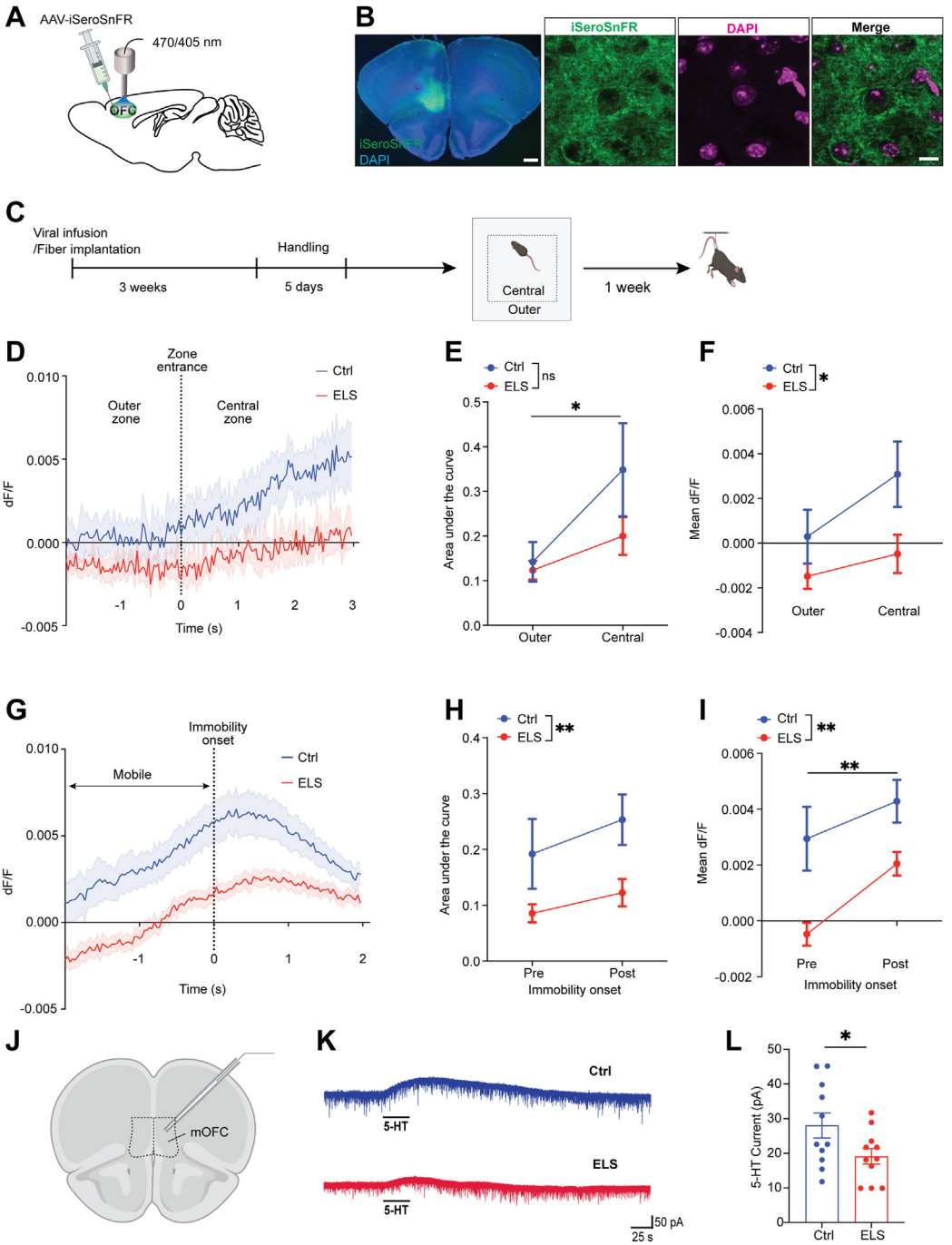

Figure 5. ELS leads to reduced 5-HT release in the mOFC under aversive conditions. **(A)** Schematic showing AAV2/9-CAG-iSeroSnFR-NLG infusion and fiber optic implantation in the mOFC. **(B)** Representative images showing iSeroSnFR (green) expression in the mOFC. Section is co-labeled with DAPI. **(C)** Experimental scheme showing viral infusion and fiber implantation followed by handling before open field test and TST. **(D)** Averaged iSeroSnFR signal 2 seconds before and 3 seconds after the mouse transitioned from the outer zone to the central zone (Ctrl: $n = 7$; ELS: $n = 9$). **(E)** Area under the curve of the iSeroSnFR signal in the outer and central zones. 5-HT is released in the mOFC after Ctrl and ELS mice entered the central zone (two-way ANOVA; effect of group: $F_{1,28} = 2.2$, $p = .15$, effect of zone: $F_{1,28} = 6.31$, $p = .02$). **(F)** Mean amplitude of the iSeroSnFR signal (dF/F) in the outer and central zones. ELS mice exhibit significantly lower 5-HT release in the mOFC in the transition from outer to central zone (two-way ANOVA; effect of group: $F_{1,28} = 6.9$, $p = .01$, effect of zone: $F_{1,28} = 3.48$, $p = .07$). **(G)** Averaged iSeroSnFR signal 2 seconds before and after immobility onset in the TST (Ctrl: $n = 7$; ELS: $n = 9$). **(H)** Area under the curve of the iSeroSnFR signal before and after immobility onset in the TST is significantly lower in ELS mice (two-way ANOVA; effect of group: $F_{1,28} = 9.91$, $p = .004$; effect of mobility: $F_{1,28} = 1.7$, $p = .2$). **(I)** Mean amplitude of the iSeroSnFR signal (dF/F) before and after immobility onset in the TST is significantly lower in ELS mice (two-way ANOVA; effect of group: $F_{1,28} = 16.82$, $p < .001$; effect of mobility: $F_{1,28} = 7.9$, $p = .009$), suggesting reduced 5-HT release. **(J)** Slices comprising the mOFC were used for patch clamp recordings. **(K)** Representative voltage-clamp recordings from mOFC neurons of a Ctrl and an ELS mouse

ELS mice, suggesting an anxiolytic effect (Figure 6F, G; Figure S6A, B in Supplement 1). In contrast, in female ELS mice, we did not obtain a difference between ChR2 (channelrhodopsin-2) and mCherry groups (Figure 6H, I; Figure S6C, D). We also did not obtain any changes in locomotor activity upon blue light stimulation in female and male ChR2 and mCherry mice in the open field test (Figure S6E–G).

To determine whether $DR^{5\text{-}HT} \rightarrow$ mOFC pathway stimulation affects stress-coping strategies of male ELS mice or overcomes the alterations in the active coping behavior observed in female ELS mice, we next performed the TST in the absence or presence of blue light. While there were no differences in the mobility of ELS males (Figure 6J; Figure S6H in Supplement 1), ELS females expressing ChR2 had significantly increased immobility compared with females expressing mCherry (Figure 6K; Figure S6I). To determine whether these effects are driven by stimulation, we performed planned comparisons between the groups during light-off and light-on conditions. Fisher's least significant difference test revealed a significant difference during optogenetic stimulation between mCherry- and ChR2-expressing ELS females (mCherry vs. ChR2; light off: $p = .16$, light on: $p = .02$), suggesting that ELS-induced increase in mobility was normalized by $DR^{5\text{-}HT} \rightarrow$ mOFC pathway stimulation.

Overall, stimulation of 5-HT neuron terminals in the mOFC significantly reduced the time spent in the outer zone of the open field, suggesting an anxiolytic effect in male ELS mice. In females, the absence of an anxiolytic effect suggests that ELS-induced anxiety-like behavior may be influenced by different circuits.

DISCUSSION

5-HT plays a critical role in socioemotional regulation; however, which neural circuits are involved in ELS-induced emotional dysregulation remains to be determined. Here, we showed that ELS disrupts the functional connectivity of the RN and alters 5-HT neuron activity in response to aversive stimuli. Further interrogation of two emotionally salient regions that receive dense 5-HT innervation revealed a significant stress-induced increase in OFC activity in ELS mice, without concomitant changes in the activity of CeA. Disrupted 5-HT release in the mOFC of ELS mice under threat coincided with a reduction in the physiological 5-HT response of mOFC pyramidal neurons.

ELS is associated with structural and functional alterations to frontolimbic connectivity (43–45). In individuals with a history of ELS, functional connectivity is disrupted in PFC, insular cortex, and amygdala (46,47) [reviewed in (48)] and the default mode network (49–51), which is implicated in stress-related disorders (52). Diffusion tensor imaging in LBN-exposed mice identified altered structural connectivity between the DRN and limbic regions in males during adulthood (53). Moreover, reactivation of ELS-susceptible corticolimbic regions enhances stress susceptibility during adulthood (54). Here, we examined the functional connectivity of the RN with 64 cortical and subcortical regions. Our data identified a shift toward anticorrelated functional connectivity of the RN in response to ELS, with this effect being most prominent in male mice. While this analysis does not delineate the underlying mechanisms that result in the change in anticorrelated activity (i.e., changes in inhibition vs. excitation in different regions), it strongly points to alterations in the coordination of activity between the RN and numerous other brain regions. Decreased correlation coefficient between ELS mice and controls was evident across most regions paired with RN (42/64 in females, 58/64 in males). Among these changes, reduced communication between the OFC and RN was particularly significant, as these regions share reciprocal structural connections, through which the OFC contributes to the regulation of 5-HT inputs throughout the forebrain (55). It is worth noting that the medial PFC also shares this relationship with the RN, and top-down control of the RN and amygdala through medial PFC connections has a critical role in the modulation of stress-coping behavior. While acute stress induces a transient increase in PFC 5-HT levels, depletion of 5-HT in the medial PFC reduces stress-induced passive coping through GABAergic (gamma-aminobutyric acidergic) transmission in the amygdala (56). Further studies are needed to dissect the contributions of each of these pathways to behavioral changes observed with ELS.

In addition to its role in affective regulation, 5-HT is involved in the modulation of punishment responses, with impaired 5-HT signaling interfering with behavioral suppression (57,58) and aversive processing (59). Heterogeneous 5-HT neurons show mixed responses to punishment (30,60). Stimulation of this mixed population increases active coping behavior in male rodents (61). However, 5-HT neuron population activity is influenced by environmental valence (33) and encodes aversive states differentially (29,30,33). Optogenetic stimulation of 5-HT neurons reduces mobility in the open field test, while increasing movement in the TST, indicating that the heterogeneous 5-HT neuron population exhibits discernible activity patterns when confronted with avoidable or unavoidable stress. This may indicate an adaptation of 5-HT neural response to differential stress-coping mechanisms. It may also suggest that threats modulate the activity of projection-specific 5-HT subpopulations differentially. For instance, decreased 5-HT signaling in the ventral hippocampus of male mice is linked to heightened active coping responses to stress, without affecting anxiety-like behavior (62). Acute footshock reduces Ca^{2+} response in the population of 5-HT neurons projecting to the OFC, while those projecting to subcortical regions respond to threat with an initial elevation in Ca^{2+} signal (30). Our data showing a selective disruption in the footshock-induced inhibitory response of 5-HT neurons suggests a subcircuit-specific impairment in ELS mice in response to

showing the outward inhibitory current in response to 5-HT application. **(L)** Amplitude of the outward inhibitory 5-HT current is significantly lower in ELS mice compared with controls (4 Ctrl male mice, $n = 11$ neurons; 3 ELS male mice, $n = 11$ neurons) (unpaired t test, $p = .048$). Data represent mean ± SEM; $^{*}p < .05$, $^{**}p < .01$. ANOVA, analysis of variance; Ctrl, control; ELS, early life stress; mOFC, medial orbitofrontal cortex; ns, nonsignificant; TST, tail suspension test.

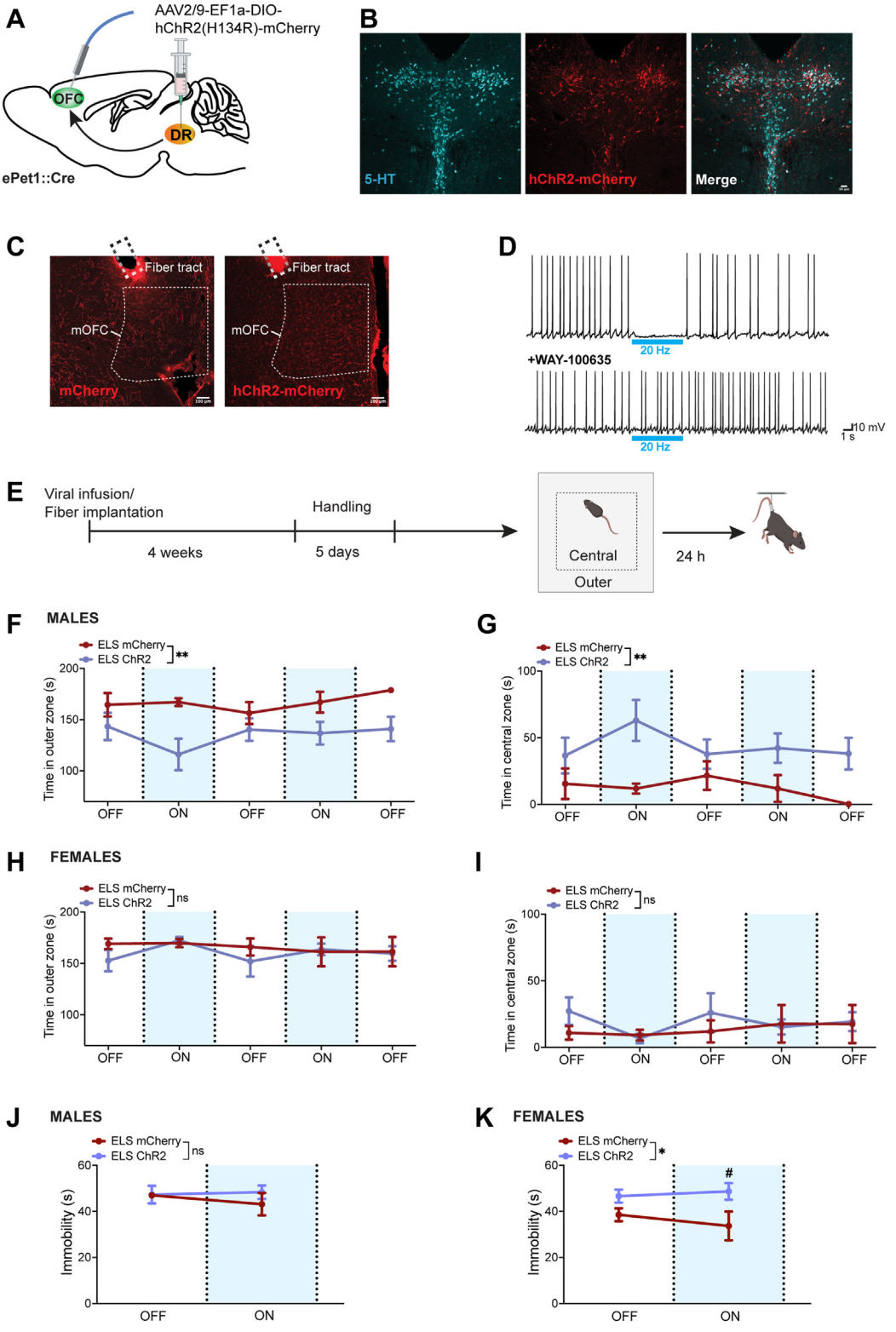

Figure 6. Optogenetic stimulation of 5-HT terminals in mOFC improves anxiety-like behavior in male ELS mice. **(A)** Schematic showing AAV2/9-EF1a-DIO-hChR2(H134R)-mCherry infusion in DR and fiber optic implantation in mOFC. **(B)** Representative images showing 5-HT (cyan) and hChR2-mCherry (red) expression in DR. Scale bar = 50 μm. **(C)** Representative images showing mCherry-labeled 5-HT neuron processes in the mOFC from mice expressing

threat. In line with this, we observed elevated footshock-induced activity of the OFC in ELS mice, indicating that this region is more sensitive to negative valence. ELS is associated with reduced OFC cortical thickness in offspring observed many years later (63,64), in association with elevated behavioral symptoms (65). Our findings offer a mechanistic insight into the potential role of the OFC in mediating emotional symptoms following early life adversities.

To our knowledge, ours is the first study that reports the effects of 5-HT neuron terminal stimulation in OFC in ELS mice. This had an anxiolytic effect in male ELS mice, but was not sufficient to improve anxiety-like behavior of ELS females. The $DR^{5\text{-}HT} \rightarrow$mOFC pathway stimulation, however, restored the exacerbated active coping strategy observed in females during TST. Although generally deemed a depression-like behavior, passive coping may be advantageous during inescapable stress by preserving energy (66). DRN 5-HT neuron stimulation has been associated with enhanced behavioral inhibition and patience via modulation of OFC activity (67). Cortical $5\text{-}HT_{1A}$ receptor signaling is critical for modulating anxiety and stress-coping behaviors (68), while the absence of $5\text{-}HT_{1A}$ receptors has been associated with decreased immobility (69). Increased passive coping may therefore indicate an improved capacity to tolerate adverse conditions by regulating the $5HT_{1A}$ receptor–mediated inhibitory tone in the mOFC of female mice. Exploring the impact of ELS-induced deficits in 5-HT signaling in the OFC on additional behaviors modulated by this region, including impulsivity, cognitive flexibility, and reward processing, could yield additional mechanistic insight into the broader effects of 5-HT dysfunction and its contribution to behavioral deficits associated with ELS.

ELS is a strong predictor for the development of affective disorders. A higher incidence of these disorders is diagnosed in women compared with men, but the rate of suicide and comorbid addiction is higher in men, suggesting differential sex-dependent coping mechanisms (70). Research exploring the role of sex as a biological factor in the long-term deficits in brain circuits associated with stress disorders has been limited. Recent studies reported early trauma-induced susceptibility to cognitive dysfunction, brain structural alterations (71), and disrupted threat response in males only (72,73), while emotional changes have been detected in both sexes. This is congruent with our current findings in which we have observed comparable behavioral deficits in both sexes and exacerbated functional connectivity changes following threat in males. Further dissection of these networks in response to different behavioral challenges will shed light on the mechanisms underlying susceptibility triggered by ELS in both males and females (74).

In summary, we present a novel mechanism for the long-term impact of ELS-induced disruptions on brain circuits that are modulated by 5-HT inputs. Our findings reveal ELS-induced impairments in the DRN 5-HT→OFC pathway. While our network connectivity findings implicate additional pathways impaired by ELS, the DRN 5-HT→OFC pathway represents a promising target for therapeutic intervention, given its critical role in reward and emotional regulation (75–78). Our findings highlight the potential of combining targeted stimulation and pharmacotherapies to improve 5-HT neurotransmission as a promising approach for treating emotional dysregulation that arises from childhood stress.

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DS, RR, and ME-P designed the experiments and wrote the manuscript. NFJ and MT performed the surgeries. RR and ME-P performed the behavioral, photometry, and optogenetic experiments and performed the histological procedures. KA, DJT, and JRE performed the network analysis. YR contributed to the photometry, and NSK contributed to the behavioral experiments. NR performed the electrophysiological experiments and analysis.

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mCherry or hChR2-mCherry. Scale bars = 100 μm. **(D)** Representative current clamp traces from mOFC pyramidal neuron showing inhibition of action potentials upon blue light (470 nm, 20 Hz, 10 ms) (top) stimulation and the absence of light-induced inhibition after application of the 5-HT1A receptor blocker WAY-100635 (bottom). **(E)** Experimental paradigm for optogenetic stimulation during open field test and tail suspension test. **(F, G)** Optogenetic stimulation of 5-HT terminals in mOFC decreases the time in the outer zone and increases the time in the central zone of the open field test in male ELS mice (mCherry: $n = 4$; ChR2: $n = 6$) (two-way ANOVA; outer zone, effect of group: $F_{1,40} = 16.59$, $p = .0002$, effect of time: $F_{4,40} = 0.62$, $p = .65$; central zone, effect of group: $F_{1,40} = 16.59$, $p = .0002$, effect of time: $F_{4,40} = 0.59$, $p = .67$). **(H, I)** No effect of optogenetic stimulation in the time spent in outer or central zones of the open field test in female ELS mice (mCherry: $n = 4$; ChR2: $n = 6$) (two-way ANOVA; outer zone, effect of group: $F_{1,40} = 0.77$, $p = .39$, effect of time: $F_{4,40} = 0.47$, $p = .76$). **(J)** Optogenetic stimulation of 5-HT terminals in mOFC does not lead to a change in mobility of male ELS mice in tail suspension test (mCherry: $n = 4$; ChR2: $n = 6$) (two-way ANOVA; outer zone, effect of group: $F_{1,16} = 0.58$, $p = .46$, effect of time: $F_{1,16} = 0.15$, $p = .71$). **(K)** Increased immobility in hChR2-mCherry-expressing female ELS mice in tail suspension test during optogenetic stimulation of 5-HT terminals in mOFC (mCherry: $n = 4$; ChR2: $n = 6$) (two-way ANOVA; effect of group: $F_{1,16} = 8.49$, $^{*}p = .01$, effect of time: $F_{1,16} = 0.12$, $p = .73$). This difference was significant only in the light-on condition (Fisher's least significant difference; mCherry vs. ChR2; light off: $p = .16$, light on: $^{\#}p = .02$). ANOVA, analysis of variance; DR, dorsal raphe; ELS, early life stress; mOFC, medial orbitofrontal cortex.

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