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Elevated fear responses to threatening cues in rats with early life stress is associated with greater excitability and loss of gamma oscillations in ventral-medial prefrontal cortex

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Abstract

Stress experienced early in development can have profound influences on developmental trajectories and ultimately behaviors in adulthood. Potent stressors during brain maturation can profoundly disrupt prefrontal cortical areas in particular, which can set the stage for prefrontal-dependent alterations in fear regulation and risk of drug abuse in adulthood. Despite these observations, few studies have investigated *in vivo* signaling in prefrontal signals in animals with a history of early life stress (ELS). Here, rats with ELS experience on PND3-5 were then tested on a conditioned suppression paradigm during adulthood. During conditioned suppression, electrophysiological recordings were made in the ventral medial prefrontal cortex (vmPFC) during presentations of a fear-associated cues that resolved both single-unit activity and local field potentials (LFPs). Relative to unstressed controls, ELS-experienced rats showed greater fear-related suppression of lever pressing. During presentations of the fear-associated cue (CS+), neurons in the vmPFC of ELS animals showed a significant increase in the probability of excitatory encoding relative to controls, and excitatory phasic responses in the ELS animals were reliably of higher magnitude than Controls. In contrast, vmPFC neurons in ELS subjects better discriminated between the shock-associated CS+ and the neutral ("safe") CS- cue than Controls. LFPs recorded in the same locations revealed that high gamma band (65-95 Hz) oscillations were strongly potentiated in Controls during presentation of the fear-associated CS+ cue, but this potentiation was abolished in ELS subjects. Notably, no other LFP spectra differed between ELS and Controls for either the CS+ or CS-. Collectively, these data suggest that ELS experience alters the neurobehavioral functions of PFC in adulthood that are critical for processing fear regulation. As such, these alterations may also provide insight into to increased susceptibility to other PFC-dependent processes such as risk-based choice, motivation, and regulation of drug use and relapse in ELS populations.

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Introduction

It is routine for individuals to experience a variety of stressors throughout their life, and the great majority are able to cope with these events and move on with minimal negative consequences on mental health. Likewise, while a large proportion of Americans routinely use psychoactive drugs recreationally, only a small proportion of these individuals will later go on to develop clinically-significant levels of drug abuse or addiction. The tipping-point between these outcomes – i.e., resumption in otherwise typical mental health outcomes, versus persistent pathologies like post-traumatic stress disorder (PTSD) or addiction – likely involves the interaction of multiple risk factors that may predispose the brain for these states. Of these, Early Life Stress (ELS) has been identified as a particularly potent risk factor due to the insults and stressors occurring during critical developmental time windows in brain maturation.

In human populations, ELS is a result of neglect, abuse and/or trauma experienced before the age of 18. Child Protective Services investigated 3.5 million cases of possible child maltreatment in 2018, an 8% increase from 2014 in the United States. However, estimating the prevalence of ELS is difficult as most cases are unreported. Human and animal studies refer to perinatal stress as exposure to severe and/or chronic stressors during prenatal and early postnatal life. While the definition is broad, it aims to highlight a critical period of growth, organogenesis and brain development in which the fetus or child are highly vulnerable to insult. During this period, exposure to stress during early life is associated with higher rates of chronic illness such as diabetes, obesity, cardiovascular, gastrointestinal and respiratory illness as well as autoimmune disorders (McEwen, 2003; Taylor, 2010).

In addition to these somatic responses, ELS can produce devastating consequences on the developing central and peripheral nervous system. Autonomic, cognitive and emotional

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dysregulation are common in individuals exposed to perinatal and ELS, and underlie most forms of mental illness (Lupien et al., 2009; McEwen, 2003). The prevalence of ELS in mental illness is alarmingly high such that 50% to 64% of patients diagnosed with depression, anxiety or substance use disorders report exposure to early life adversity (Dube et al., 2003; Enoch, 2011; Vogt et al., 2016). Clinical studies have also reported a positive correlation between the intensity and duration of ELS and the number of psychopathologies an individual develops, as well as symptom severity (Carr et al., 2013). Indeed, a host of stress and anxiety disorders such as generalized anxiety disorder, panic disorder, post-traumatic stress disorder (PTSD) and obsessive-compulsive disorder have the highest rate of co-occurrence and are comorbid with substance use disorders (SUD) (Regier et al., 1990), all of which have been linked to ELS as a predictor of their development (Brady and Sinha, 2005; Brown and Barlow, 1992; Enoch, 2011; McEwen, 2003; Regier et al., 1990).

Apart from the differences in timing, the rodent and human brain follow similar patterns in which the sequence of structural development is highly conserved (Rice & Barone, 2000). Rodents exposed to ELS have reported morphological, neurochemical and behavioral alterations that parallel some of the findings reported in humans (McEwen, 2003; Weinstock, 2017, 2008). Unlike subcortical structures, in which early cellular processes such as migration, differentiation, synaptogenesis and gliogenesis are well underway, limbic structures such as hippocampus, amygdala and prefrontal cortex (PFC) are in their early stages of development at birth (Herlenius and Lagercrantz, 2004; VanTieghem and Tottenham, 2018). More importantly, the PFC is the last cortical structure to fully develop as it continues these processes into adulthood making the PFC highly susceptible to environmental insult throughout childhood (Kroon et al., 2019; VanTieghem and Tottenham, 2018). During the early stages of development, cortical and subcortical projections

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reach the PFC to ensure proper communication between the PFC and the rest of the brain, many of which introduce neuromodulatory activity into the region. These neuromodulators play critical roles in the development of PFC circuits during the first week of postnatal life. Additionally, increased glutamatergic activity during early development dominates cortical pyramidal cell transmission and is critical in synaptogenesis as well. NMDA activity is predominant and aids in fine tuning synapses and in areas of excessive activity it enables apoptosis (Herlenius and Lagercrantz, 2004; Rice and Barone, 2000). Collectively, these limbic brain regions are interconnected, and in adulthood of neurotypical animals, coordinate activity to support appropriate responses to motivated learning and cognitive flexibility.

In rodents, the PFC along the medial aspect frontal regions is typically subdivided into two functional divisions, the dorsal medial aspect (*dmPFC*; which includes the prelimbic [PL] cortex) and the ventral medial aspect (*vmPFC*; including the infralimbic [IL] cortex). Though these PFC regions lack direct homology with primate regions (Laubach et al., 2018), evidence exists that functional and developmental overlap between rodent vmPFC and similar human prefrontal regions such as Brodmann's area 25. For example, this region has been implicated in cognitive and behavioral flexibility deficits in patients that suffer from Anxiety Disorders, ASD and SUD (Greenberg et al., 2013; Jackson et al., 2016; Myers-Schulz and Koenigs, 2012), as well as decreased activity during fear generalization tasks (Greenberg et al., 2013). Similar activity patterns were also detected in individuals suffering from PTSD when presented with trauma associated cues (Shin et al., 2004) and extinction recall (Milad et al., 2009).

In rodents, vmPFC participates in similar functions (Giustino and Maren, 2015). Via connections with limbic targets such as the amygdala complex, the IL is involved in the consolidation, maintenance and expression of extinction learning as well as habitual behaviors

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(Quirk and Mueller, 2008). Within the domain of fear conditioning specifically, IL activity is both necessary and sufficient to support fear extinction. Stimulation of IL accelerates fear extinction (Adhikari et al., 2015; Bukalo et al., 2021; Milad and Quirk, 2002) and suppresses spontaneous recovery of fear (Kim et al., 2010), while neurons in this area increase activity during the early stages of extinction learning, with cue-elicited phasic activity emerging only after extinction learning has occurred (Sierra-Mercado et al., 2011a). Conversely, pharmacological or optical silencing of this IL pathway in fear extinction learning results in increased freezing behavior and reduced extinction rates (Adhikari et al., 2015; Gutman et al., 2017; Laurent and Westbrook, 2009).

However, ELS appears to alter these normal fear and anxiety-related processes, disrupting conditioned fear as well as decreasing exploration of open arms in an elevated plus maze (Nisar et al., 2019; Oldham Green et al., 2021; Toda et al., 2014). While recent work has provided valuable insight into the genetic and epigenetic bases for these differences particularly in the PFC (Oldham Green et al., 2021; Torres-Berrío et al., 2019), less is known about how mature neurons in ELS-experienced animals encode information about threat and safety during behavior. Using a variant of a limited bedding model of ELS (Molet et al., 2014; Walker et al., 2017), we recorded from animals who received either the ELS or Controls who were allowed to develop without stress during a conditioned suppression task. Unlike standard fear conditioning, in conditioned suppression tasks, rats decide whether to withhold motivated seeking (pressing for rewards) in the presence of threatening cues (such as shock-associated tone), producing a behavioral conflict between desire for rewards and passive defensive behaviors with would prevent seeking those goals. Here we report that ELS animals showed greater fear-suppressed motivation than Controls,

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and that during this task, vmPFC neurons displayed greater excitatory responding to fear cues, but decreased high gamma oscillations in the local field potential band.

Methods

Subjects

Subjects for this experiment were initially 26 male and female Long-Evans rats (9 male and 17 female) at the start of the experiment. All animals were bred in-house in the CU Boulder vivarium in an AAALAC-approved facility. Subjects were bred against a TH::Cre background (males were TH::Cre-positive Long-Evans [original line sourced from Rat Resource & Research Center (RRRC)] mated with female standard non-transgenic Long-Evans [Envigo]), though we did not use manipulations to selectively target TH-containing cells in this study, so cre status was not assessed in this study in group assignments. All rats were bred in-house and maintained on a 12:12 light/dark cycle (lights on 7:00 A.M.–7:00 P.M.) and all testing occurred during the light phase.

The behavioral procedures occurred in two distinct periods. During the first ("early life") phase of the experiment (PND 0 - PND 21), dams were allowed *ad libitum* access to enriched breeder chow (Teklad) and water in the home cage, while pups were allowed *ad libitum* access to nursing. During the second ("adulthood") period for the original pups, rats were first tested under ad libitum conditions (PND180), and afterwards (approximately PND 180-PND 320) restricted to 95% of their free feed weight receiving 10-15g of standard laboratory chow (Teklad) provided directly in their home cage after daily test sessions. Note that while developmental assays reflected all subjects described, due to an animal care error, all but three of the males in this study were sacrificed just prior to reaching adulthood and subsequent testing.

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During the pre-weaning period (PND 0-PND 21), the dam and pups were housed in a plastic container (48cm (l) X 26cm (w) X 20cm (h)) with approximately 2cm of wood shaving bedding and a laboratory paper twists for enrichment and nesting material. For the remainder of the experiment the home cage consisted of a plastic container (48cm (l) X 26cm (w) X 20cm (h)) with approximately 2cm of bedding. All procedures were performed in accordance with University of Colorado Institutional Animal Care and Use Committee guidelines for the humane use of laboratory rats in biological research.

Behavior

Early life stress (wet bedding and nesting material)

Breeding pairs of rats were originally intended to be used for a different study involving TH::cre lines. Four male/female pairs were created on the same day, and after mating, pregnant females were isolated to gestate without males present. All females used in this study were new to the colony after arrival from the vendor, and as such, the resulting litters were born to first-time dams. Of the four cages, two suffered a water supply malfunction **(**Hydropac Alternative Watering System), in which both thin-plastic hydropacs that provided water to the dam leaked and flooded the cages, resulted in exposure to wet and cool bedding (2 cm) and nesting material for the dam and pups for approximately 24-72hrs (PND 2-PND 5). On PND 5, the dam and their pups were returned to normal bedding conditions. In the other two cages, dams produced pups that were left undisturbed with normal bedding and nesting conditions throughout development. Based on these conditions, we were unsure whether this wet/cold setting early in life might alter aspects of brain development. As such, pups who experienced the wet/cold cage were designated the Early Life Stress (ELS) group, while the non-stressed pups born at the same time in the vivarium (within one

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203 week) comprised the Control group. Animals were pair-housed during the post-weaning period
204 into adulthood, and were only separated following intracranial implants just prior to Conditioned
205 Suppression training (see below)
206
207 *Early Life: Juvenile Social Exploration*
208 Social exploration tests were conducted at PND 180 as described previously (Christianson
209 et al., 2011). For this test, each subject was placed in plastic tub cage (48cm X 26cm X 20cm) that
210 contained 2 cm of fresh bedding located in a designated testing room for a 1-hour habituation
211 period. A novel Long-Evans juvenile rat (target) was then introduced into the cage for 3 min. The
212 behavioral response of the subject animals (ELS/Controls) to the target juvenile was recorded with
213 a video camera placed above the cage. The behavior was then assessed by measuring total duration
214 and frequency of social exploration. All videos were scored in a blinded and randomized manner.
215 *Adulthood: Conditioned Suppression*
216 As adults, rats were trained in a Conditioned Suppression paradigm. This task consisted of
217 a sequence of three phases: instrumental conditioning [Context A], auditory fear conditioning
218 [Context B], and finally, fear-conditioned suppression test [Context A]. The specific approaches
219 are explained in detail below.
220 Instrumental Conditioning: Lever press was established to assess motivation to seek
221 rewards. On the first day of training, rats (n=16) were introduced to a large operant chamber
222 (Context A: 60cm (w) X 56cm (l) X 36cm (h), smooth Plexiglas floors; MED Associates) where
223 they were first magazine trained to obtain food (45mg raspberry-flavored grain pellets, Purina Test
224 Diet) randomly delivered to a centrally-located foodcup on average about every 60s schedule.

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On the subsequent days, a lever was extended and rats could press the lever and obtain food. Each session ended when the rat pressed the lever enough times to deliver 25 pellets or 30min, whichever occurred first. If a rat received the 25 pellets within the 30min session limit, on the following day, the subject was promoted to the next reinforcement schedule. Rats were first trained on an FR1 schedule of reinforcement (each press produces a pellet), followed by variable interval schedules of VI-5 (i.e., the first press in each 5-sec bin reinforced), then VI-15, VI-30 and finally VI-60. Following completion of the VI-60 schedule, rats were implanted with bilateral electrophysiological arrays (see below), then allowed at least 7d to recover. Following recovery, rats were retested in the VI-60 schedule to re-establish stable pressing behavior. For rats who failed to perform adequately at the VI-60 schedule, retraining at denser reinforcement schedules until they could once again advance to stable completion of the VI-60 schedule over three consecutive days.

Fear Conditioning: On the day following completion of the last VI-60 schedule of pressing, rats were trained in a standard tone-shock fear conditioning paradigm consisted of a single 49-min session. In this task, subjects were tested in a novel chamber (Context B: 43cm X 43cm X 53cm, stainless steel grid floor; MED Associates) that was located in a different location in the research facility from the original Instrumental Context A. The first 5 min of the session consisted of a habituation phase where no cues were presented followed by a randomized presentation of a total of 14 trials with a 180s ITI (7 CS+ trials: 30s tone (5000Hz, 80dB) co-terminating with a 0.8mA footshock delivered through the stainless floor grid bars, and 7 CS- trials: 30s tone (3000 Hz, 80dB) presented without any programmed consequences).

Conditioned Suppression: The day after Fear Conditioning training, test subjects were returned to the original Instrumental Context A chamber. As in prior instrumental testing sessions,

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a lever was presented and presses were reinforced with 45mg pellets on a VI-60 schedule. However in the Conditioned Suppression sessions, after an initial 5min habituation phase where no cues were presented, rats received random presentations of either the previously shock-predictive CS+ cue (30s high tone; 5000 Hz, 80dB; n=7 presentations) or the neutral CS- cue (30s low tone; 3000Hz, 80dB; n=8 presentations). Note that in this Context A, auditory cues were presented without shock under extinction conditions. Cues were presented with an ISI on average of 150s throughout the session. The number of lever presses during baseline period (no cues present) was compared to pressing during both the CS+ and CS- period to assess the degree of cue-elicited suppression of instrumental activity. Rats received three consecutive Conditioned Suppression sessions to assess the rate of recovery of instrumental pressing with extinction of the fear response.

Electrophysiological Recording

Single-unit recordings were acquired during Conditioned Suppression test sessions. Using Plexon Omniplex systems (Plexon, Dallas TX) with a sampling rate of 40 kHz, analog voltages recorded at the site of wires relative to a ground wire were amplified with a unity gain head stage. Neural activity was then digitized and low-pass filtered to remove artifacts through a Plexon MiniDigiAmp A/D converter and collected with the OmniPlex Server on a wideband spectrum. This signal was then passed through a high-pass filter to capture unit spikes, while a low-pass filter captured local field potentials (LFPs). OmniPlex received synchronized TTL inputs from the MED Associates system running the test chambers to capture real-time behavioral events (including experimenter-delivered events like cues and reward delivery, and subject-generated inputs like lever presses), allowing perievent analysis of neural activity and LFP power.

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Surgery

Electrophysiological Recordings

A subset of subjects (n = 13 rats; 10 female, 3 male) underwent bilateral electrode implantation surgery (n= 7 ELS [6F/1M], 6 CTL [4F/2M]). Stereotaxic surgery was performed under isoflurane anesthesia (2–5%) using aseptic techniques. For each surgery, rats were secured in a stereotaxic apparatus (Kopf) using blunt earbars. Hair on the scalp was removed and the underlying skin scrubbed with two sets of alternating washes of Betadine scrub and 70% ethanol. Optical ointment (Vaseline) was applied gently to protect the eyes. A midline incision was made with a scalpel, and the scalp and underlying fascia retracted laterally, which was then secured with hemostats clamped to the fascia. The locations of Bregma and lambda were marked with a Pilot Precise V5 pen. A probe attached to the stereotaxic was used to measure the DV and ML deviations of Bregma and lambda; deviations of more than 0.1mm were adjusted until the head was level. Coordinates for array implants were generated from an atlas from Paxinos and Watson (2004) for infralimbic cortex (AP, +2.7; ML, ±0.5), and then holes drilled using a dental burr over the location. At each of these insertion sites, the underlying dura was retracted to ensure the wires were inserted directly into brain tissue. In addition, holes were drilled for skull screws (typically three on each hemisphere) and the location of the ground wire (one each hemisphere).

Once holes were drilled, the skull screws were inserted. After this, each array was inserted (AP, +2.7; ML, ±0.5; DV, −5.0 mm), with the left inserted first. Arrays consisted of two 8-wire electrode arrays (circular array surrounding an optical fiber; each wire consisting of a 50-µm dia Teflon-coated stainless-steel wire spaced 500 µm apart; NM Labs, Denison, TX). Arrays were lowered slowly (approximately 0.5mm/min) to the final recording location where the insertion hole and wires were secured with a light application of dental acrylic. After this, the ground wire

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was wrapped around the posterior skull screw, then inserted into the brain at the ground wire hole. The ground wire was then secured with an application of dental acrylic at the insertion site. Prior to use, array wires are kept in alignment between the base of the connector (Omnetics micro) to near the wire tips with polyethylene glycol (PEG); once inserted, a gentle sterile saline wash was used to dissolve the PEG substrate away from the wires. Once dissolved, the flex array was gently lowered to as to coil the remaining wire, and then secured with dental acrylic. This was then repeated on the right side, with a specific mount used to ensure the two array connectors were spaced correctly for the headstage tethers that would be used later on the recording rigs. Rats received intramuscular injections of the antibiotic Baytril and the NSAID analgesic Meloxicam-SR at the end of surgery. Rats were given a 7-day post-surgery recovery period before conditioned suppression training began.

Perfusion and Histology

Following the final behavioral test, rats were deeply anesthetized using isoflurane 4% and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Electrode placements were marked by passing current from a 9V battery through each electrode wire. Brains were postfixed in 4% paraformaldehyde for at least 12h, followed by 36-48h in 20% sucrose as a cryoprotectant, then stored at −80°C. Tissue was sectioned at 20 μm and mounted onto SuperFrost Plus slides (Fisher Scientific) using a cryostat at -20°C and imaged using a light microscope (Leica) to confirm electrode placement.

Data Analysis

Behavioral Analysis

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During Early Life, behavior was assessed for pup weight, ultrasonic vocalizations, and juvenile social exploration. These data were analyzed with a between-subjects (unpaired) t-test using ELS and unstressed Controls as the factors of interest.

During Adulthood, behavior for Conditioned Suppression was measured by using a suppression ratio. Presses made during the 30-sec cue presentation (CS) was compared the 30-sec period immediately prior to the cue onset (BL). The suppression ratio for each stimulus type (i.e., CS+ or CS-) was calculated as:

$$Suppression\ Ratio = \frac{(CS - BL)}{(CS + BL)}$$

This produces a range of scores from -1 to +1, with -1 being total suppression of pressing during the cue and 0 reflecting no difference in pressing during the cue relative to the baseline. Differences between groups for behavioral suppression were determined using two-way ANOVA using the factors of Day (Days 1-3) and Stress (ELS vs unstressed Controls) as the variables of interest. Tukey's HSD test was used for post hoc comparisons.

Single Unit Electrophysiological Analysis

Putative single units were sorted for each channel (wire) using principal component analysis clusters based on waveform similarity (Offline Sorter; Plexon). Unit clusters were then subject to secondary confirmation using auto-correlated firing properties. Auto-correlated firing histograms typically contain a "notch" at the 0 point indicative of a biologically-relevant refractory period for action potential generation (typically at least +/- 4 ms) Putative cells that showed significant numbers of spike events in this refractory period were rejected as units as being biologically implausible, and were not subsequently analyzed.

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For perievent analysis, data were binned into 200ms blocks and averaged across events within a session. The perievent firing rate was then z-transformed based on mean and standard deviation of the average perievent activity. Thus, the z-normalized firing rate for each bin was calculated as:

$$z_{Bin} = \frac{(FiringRate_{Bin} - Mean\ BL\ Firing\ Rate)}{StDev\ BL\ Firing\ Rate}$$

To ensure relatively uniform distributions of z-normalized firing, units with activity of less than 0.5Hz were excluded from subsequent analysis. Within the remaining units, averaged populations were grouped by generally excitatory (firing rate greater than 0.5z within 1s after cue onset) or generally inhibitory (firing less than -0.5z within 2s after cue onset). Based on this, we assessed two components of perievent cue firing. The first is the relative proportion of cells that exhibited generally excitatory (>0.5z), generally inhibitory (< -0.5z) or non-phasic relative to cue onset. These proportions were compared using chi square analysis. The second quantifies the peak firing during these onset periods for each cell. These were assessed using three-way ANOVAs with Stress (CTL vs ELS), Cue (CS+ vs CS-) and Epoch (Baseline vs Cue Onset) as factors.

Local Field Potential Analysis

LFP data generated spectrograms from 1-120 Hz perievent aligned to the CS+ and CS- cues. Spectrograms included a 5sec baseline followed by a 30s cue presentation and a 5-sec post-cue period, averaged into 200ms bins. Prior to fast Fourier transform (FFT), spectrograms were mean background subtracted, then normalized by the log of the Power Spectral Density (dB). From these spectrograms, specific frequencies were selected based on their established importance in circuit signaling: Delta (1-4 Hz), Low Theta (5-8 Hz), High Theta (9-14 Hz), Beta (15-22 Hz), Low Gamma (23-55 Hz), and High Gamma (65-95 Hz). The average power in these bands were

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then z-normalized by the average and standard deviation of the 5sec baseline period prior to cue onset, then applied to each 200ms bin throughout the perievent trace (similar to that described above for neural activity normalization).

For stress-related comparisons, for each spectrum, the subject's baseline and average power during the cues (CS+ and CS-) was assessed separately for all days of the Conditioned Suppression tasks. Note that the averaged power during the cue period excluded the first 400ms of activity, but did include the rest of the cue period. These were assessed using three-way ANOVAs with Stress (CTL vs ELS), Cue (CS+ vs CS-) and Epoch (Baseline vs Cue Onset) as factors.

Results

Behavior

Early Life Stress Impairs Development

Pups were weighed six days following birth (PND6). We found that stress had a significant negative impact on weight gain, with animals in the ELS group showing reliably lower weights than Controls, $t_{24} = 15.85$, $p < 0.0001$ (Figure 1A).

ELS Reduces Social Behaviors

After growing to adulthood in the vivarium (but prior to any experimental conditioning), adult rats were assessed in a juvenile social interaction (JSI) task on PND180 to assess social behaviors and anxiety-related phenotypes. Rats in the ELS group generally showed a decrease in social behaviors compared to Controls. While the total time spent sniffing the juvenile

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conspecific was marginally decreased in the ELS group (t_7 = 2.32, p=0.052; Figure 1B, left), the number of interaction bouts initiated by the ELS subjects were significantly lower than unstressed Controls, t_7 = 3.14, p=0.017; Figure 1B, right).

Figure 1 about here

ELS Abnormally Suppresses Motivated Seeking Under Threat

In the acquisition phase of instrumental learning, ELS appeared to have no effect on the motivation to press for food. Rats in the ELS group showed a similar ability as Controls on the last three days of each schedule to press for the food, and likewise to increase the number of presses per reward delivered based on the schedule requirements (Figure 1C). These observations produced a significant main effect of Schedule, $F_{2,223}$ = 200.7, $p < 0.001$. This effect was almost exclusively due to a linear increase in press rate per reward earned across the decreasing reinforcement schedule, as a linear contrast on these data was significant, $F_{1,223}$ = 274.1, $p < 0.001$ and accounted for 82% of the main effect variance. However, there were no effects of Stress or any interactions of Stress by any other factor (Schedule, Day) (all $F < 1$).

Following this, rats were returned to the original context for the Conditioned Suppression task. Here, rats were reinforced on a VI60 schedule while receiving presentations of the CS+ (fear-associated cue; n=8) or neutral CS- (n=8). Because no shocks were delivered in this context, we repeated this Suppression paradigm for three consecutive days to assess the rate of fear extinction. For average pressing within each session, both groups showed suppression of lever presses during the presentation of the CS+, though ELS subjects were more suppressed than Controls (main effect Stress, $F_{1,16}$ = 5.21, p=0.047; Figure 1D). This suppressive effect in the ELS animals was limited to the CS+ (interaction of Stress X Cue, $F_{1,16}$ = 10.54, p=0.005), with ELS showing reliably greater

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suppression than controls during the CS+ (Tukey, p=0.005), but no differences between the CS- (Tukey, p=0.99).

Finally, we assessed the degree of successful extinction by assessing whether the suppression ratio was reliably negative (i.e., still suppressed) on each session. Controlling for multiple comparisons (Bonferroni), we found that in Controls, suppression for the CS+ was reliably below 0 on Day 1 (p<0.001), but not on Day 2 or Day 3. In contrast, ELS rats showed suppression during the CS+ that was reliably below 0 on all three days (Day 1: p<0.0001; Day 2: p=0.005; Day 3: p=0.01). Overall, these data indicate that relative to Controls, ELS animals display greater suppression of motivated behavior to fear-related stimuli that is more resistant to extinction than in Controls. However, ELS rats do not appear to show generalized fear, as they are adept at discriminating between fearful and "safe" stimuli; indeed, even better than Controls.

ELS increases the rate of excitatory responses to fear cues in vmPFC neurons

Recordings of single unit activity were conducted during Conditioned Suppression in both Controls (n=6) and ELS (n=7) subjects. From these recordings, we identified n=129 neural units in the Controls and n=191 in the ELS subjects in histologically-confirmed locations in the vmPFC (Figure 2).

Figure 2 about here

Z-normalized firing rates were then aligned by their phasic response to the onset of the fear-associated cues (CS+ and CS-) by taking the average Z score during the first 1sec following cue onset. Data were considered generally excitatory if they exhibited an increase in firing greater

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than +0.5z relative to baseline, while inhibitions were those with a phasic response less than -0.5z. Data from all recorded cells relative to CS+ onset are shown in Figure 3A (Control) and Figure 3B (ELS). Population responses to the CS+ cue in the Controls were biased towards inhibitory signaling with 36.7% of cells demonstrating phasic inhibitions and 28.9% displaying excitations; 34.3% of cells were non-phasic in either direction. In contrast, ELS neurons showed the opposite pattern, as these units were almost twice as likely to display an excitatory response (50.5%) than an inhibitory response (25.5%) to the CS+ cue. ELS neurons also had slightly fewer cells that were non-phasic (24.0%). This shift from inhibitory to excitatory response to the CS+ between groups was significantly different, $\chi^2_1 = 10.96$, $p = 0.0009$ (Figure 3B). Notably, these EXC/INH/non proportions were quite stable by groups over days, with Controls showing generally more inhibitory responses than excitations to the CS+, and ELS showing the opposite pattern (*Controls*: Day 1 - 29% EXC, 35% INH; Day 2 – 32% EXC, 53% INH; Day 3 – 33% EXC, 27% INH; *ELS*: Day 1 - 56% EXC, 20% INH; Day 2 – 48% EXC, 31% INH; Day 3 – 40% EXC, 21% INH; all χ^2 comparisons between day, $p>0.20$). In contrast to the fear-associated CS+, the relative proportion of excitatory and inhibitory response to the CS- cues was not different between groups (Control: 29.2% excitatory vs 32.1% inhibitory; ELS: 41.7% excitatory vs 34.9% inhibitory; $\chi^2_1 = 0.57$, $p = 0.45$). These data suggest that ELS experience alters the function of the vmPFC to bias neurons towards an abnormally excitatory response to threatening (but not neutral, or "safe") cues.

Figure 3 about here

ELS impairs normal shifts in extinction-related firing to the CS+ cue

Prior investigations have reliably demonstrated that vmPFC neurons are critical for mediating extinction of fear via connectivity with amygdalar structures (Adhikari et al., 2015;

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Maren and Quirk, 2004; Milad and Quirk, 2002; Sierra-Mercado et al., 2011b). Phasic perievent excitatory activity relative to the CS+ in the Controls was consistent with this established finding, demonstrating a slight increase in the magnitude of phasic activity over days. In contrast, vmPFC neurons in ELS animals showed the opposite pattern, with the greatest level of excitatory activity during the CS+ occurring on the first day and decreasing magnitude of this response over repeated days of extinction (Figure 4A). In general, the ELS animals showed a reliably higher overall excitatory phasic response to the fear cues (main effect Stress, $F_{1,133}$ = 4.77=8, p = 0.03) and an interaction of Cue (CS+ vs CS-) X Stress (ELS vs Control) X Extinction Day (1-3), $F_{2,133}$ = 3.34, p = 0.04. This interaction showed that the phasic response to the CS+ was greater in ELS than Controls on Day 1 (p = 0.005) but not on subsequent days. There were no differences to the CS- between groups on any day (Figure 4B). However, in general, CS+ elicited significantly greater activity than the CS- in both the Controls (p = 0.02) and in the ELS group (p < 0.001).

In contrast to the excitatory phasic responses, for inhibitory responses, there were no stress-related main effect, $F_{1,104}$ = 3.07, p = 0.08, though there was an interaction of Stress X Day, $F_{2,104}$ = 3.18, p = 0.046, and Stress X Cue, $F_{1,104}$ = 5.90, p = 0.017, but not Stress X Cue X Day, $F_{2,104}$ = 0.75, p = 0.47 (Figure 4C). Indeed, for planned comparisons, we found no differences in the magnitude of the inhibitory response between Controls and ELS for the CS+ cues (all p > 0.28), though there were differences between ELS and Controls for the “safe” CS- on Day 1 (p < 0.001) and on Day 2 (p = 0.04), but not on Day 3 (p = 0.75). On those same days, ELS animals showed a better ability to discriminate neural firing responses between the CS+ and CS- on Day 1 (p = 0.005), Day 2 (p = 0.001) and Day 3 (p < 0.001), whereas Controls only

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successfully discriminated between CS+ and CS- cues on Day 3 (p = 0.01) but not on Day 1 (p = 0.75) or Day 2 (p = 0.76), Figure 4D.

Figure 4 about here

ELS abolishes High Gamma LFP responses to threat cues in vmPFC

LFPs have been proposed to reflect the coherence of the aggregate voltage in a region. Given the large amount of surface area on dendritic arbors in a region relative to somatic activity, one potential interpretation of LFP oscillations is that it reflects a significant component of input to those local arbors from afferent regions. We recorded LFPs on the same wires and locations in the vmPFC as for the single-unit activity described above, and generated perievent spectrographs for defined frequency bands relative to CS+ and CS- onset during the same Conditioned Suppression sessions (West et al., 2021).

We found that ELS experience had little effect on changes in LFPs in most spectra (Figure 5). For example, in the Delta, Beta and Low Gamma frequencies, the response to the cue onset reliably decreased the power of these frequencies relative to baseline for the CS+ but not CS- (Cue [CS+ vs CS-] X Onset [baseline vs cue periods]: Delta, $F_{1,53}$ = 14.52, p = 0.0004; Beta, $F_{1,53}$ = 8.90, p = 0.004; Low Gamma, $F_{1,53}$ = 11.66, p = 0.001). However, while the LFP response to the CS+ decreased LFP power in these spectra below baseline for the CS+ (all p<0.003) but not CS-, there were no differences between Controls or ELS for either CS+ or CS- in any of these spectra (all p > 0.25). Notably, there were no main effects of Stress or interactions of Stress with other factors in the Delta, Low Theta, High Theta, Beta, or Low Gamma frequencies.

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However, ELS appeared to selectively impair the High Gamma frequency response (Figure 5, *bottom right*). In Controls, this response was a large and sustained increase in power for the duration of the CS+ cue, which then returned to baseline after cue offset. In contrast, this frequency response showed only a brief <1sec response to cue onset before rapidly returning to baseline for the rest of the CS+ cue. Quantifying the mean response during the cue period for both CS+ and CS- (Cue) for ELS and Controls (Stress) for the baseline period vs cue period (Onset), an ANOVA found a main effect of Stress, $F_{1,53} = 6.89$, $p = 0.011$, and an interaction of Stress X Cue, $F_{1,53} = 6.76$, $p = 0.012$, Stress X Onset, $F_{1,53} = 6.90$, $p = 0.011$, and Stress X Cue X Onset, $F_{1,53} = 6.76$, $p = 0.012$. Posthoc comparisons of this 3-way interaction indicated that in Controls, the High Gamma response to the CS+ was reliably higher than both its own baseline (p=0.0001) and the CS- cue (p=0.0001). The power for the CS- cue in Controls was, however, no different than its preceding baseline (p=0.99).

Figure 4 about here

For ELS animals, this selective CS+ related increase in power was eliminated; the average power of the CS+ was no different from baseline (p=0.40) nor from the CS- period (p=0.99). Consistent with this loss of High Gamma power in the ELS group, there was a significant decrease for the High Gamma band for the CS+ cue between groups (p=0.0008), but there were no differences in power between groups for the CS- cue (p=0.99). As such, ELS appears to selectively abolish a discrete component of the LFP spectra, while leaving other lower-frequency components relatively unaffected.

Discussion

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Fear is an adaptive response to potentially threatening stimuli, though the brain must be adaptive enough that fear can extinguish when threats are no longer present. Consistent with prior observations, we found that ELS experience increases the fear-related suppression of reward seeking during the fear-associated CS+ compared to Controls. However, ELS animals did not differ from controls in their responses to the "safe" CS- cue. During these conditioned suppression sessions, recordings were made in the vmPFC that permitted the recording of both single unit and LFP activity in the same location. Neurons in the vmPFC of ELS rats showed both an increase in the proportion of excitatory responses to the fear-associated CS+ cue compared to Controls, as well as an increase in overall magnitude of the excitatory phasic response to cue. In contrast, while there were no differences between ELS and Controls in inhibitory encoding of the CS+, ELS neurons were better able to discriminate between CS+ and CS- stimuli than those in Controls. Finally, LFP oscillations in the vmPFC were consistent with a selective loss of the high gamma band in ELS-experienced rats. This loss is notable in that this is the only frequency where Controls showed a phasic change in activity that discriminated between CS+ and CS- stimuli, suggesting this signal plays a potentially important role in facilitating fear discrimination and feedback during extinction. Collectively, these data are among the first to demonstrate ELS-related functional alterations in vmPFC activity and resultant changes in fear-related behavior.

In general, our finding in Controls are congruent with prior findings in neurotypical adult rats undergoing fear conditioning and extinction. Controls showed initial fear to the CS+ stimulus that resulted in a robust cessation of motivated pressing for food. However, these fear-suppressed behaviors rapidly returned to pre-suppression levels by the second day of extinction. In these same Control subjects, vmPFC activity showed an appreciable increase in phasic

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554 excitatory activity in response to the CS+ commensurate with the resumption in motivated
555 seeking behavior and extinction of the fear-related suppression, while showing reliably less
556 activity to the safer CS- cue. These data are consistent with prior work demonstrating the role of
557 IL and the vmPFC in mediating extinction through new learning (i.e., that the CS+ is now
558 associated with no-shock), and increases in excitatory activity in these regions to permit this
559 plasticity. Prior work, for example, has shown that excitatory stimulation of IL via electrical
560 current or channelrhodopsin is sufficient to expedite fear suppression and extinction (Adhikari et
561 al., 2015; Giustino and Maren, 2015; Milad and Quirk, 2002), and which persists in subsequent
562 days without the stimulation present.

563 The ELS animals showed a different pattern of results; unlike Controls, ELS animals
564 showed greater overall amounts of fear suppression, while at the same time showing increased
565 levels of excitatory responding in single units. It is essential to note that ELS animals face
566 developmental alterations compared to unstressed neurotypical controls. For example, PFC
567 regions continue to develop and integrate neuromodulatory afferents for several days (at least
568 PND16) after birth, including mesocortical dopaminergic wiring and integration with amygdalar
569 nuclei (Cunningham et al., 2008; Kalsbeek et al., 1988; Kroon et al., 2019; Yuan et al., 2021),
570 producing lasting changes in excitability and functional properties of these networks
571 (Muhammad et al., 2012; Zhang, 2004). Thus, for these ELS animals whose stress experience
572 happened during this critical developmental window, functional responses of these neurons may
573 not mirror those in neurotypical individuals. Indeed, the robust and consistent increase in
574 excitability in these neurons suggests that for ELS animals, the typical relationship between
575 greater excitability and faster extinction seen in neurotypical controls no longer holds.

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These observations argue against an interpretation of ELS inducing a hypofrontal state where extinction of threats are unable to be extinguished by descending prefrontal networks. This outcome would be consistent with some prior work showing decreased activity in human populations during reward and risk processing (Birn et al., 2017), and frankly with our *a priori* predictions for this study. However, the increased excitability suggests instead that vmPFC neurons are appropriately responding to the threat posed by the CS+ cue and are appropriately increasing activity to drive extinction, but that this activity is not sufficient to dampen fear-induced suppression as in controls. A possible interpretation for this set of results is that extinction is a process that requires both new learning (CS+ no longer predicts threat) as well as feedback to stamp in those new associations. Recent findings and models are consistent with the importance of these potential feedback mechanisms to the PFC in normal fear learning and extinction (McNally et al., 2011). For example, during processing of fear stimuli, mesocortical dopaminergic input to the PFC (Vander Weele et al., 2018) as well as amygdalar input (Burgos-Robles et al., 2017) provide event-related information about fear threats to prefrontal networks. Indeed, recent work has demonstrated that pathway-specific inputs from intercalated neurons in the basolateral amygdala to discrete components of dorsal and ventral PFC may differentially regulate feedback to gate continued fear or its extinction (Hagihara et al., 2021). If this is the case, then persistent increases in fear-associated excitability in vmPFC of ELS animals may not be due to an inability to detect threats, but rather for a PFC-amygdala network to cooperatively use error-related feedback to update cues to a new and less-threatening state. If so, then evidence should exist that ELS animals are missing arising information that could be relevant for this learning.

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598 Consistent with this interpretation, LFPs in the high gamma band were largely abolished
599 in ELS compared to controls. LFPs reflect aggregate voltage in a region, and given the density of
600 dendritic arbors relative to somas, these changes in voltages in a region may biased towards
601 reflecting afferent inputs to a region, via depolarization and hyperpolarization of dendrites
602 receiving those signals. Support for this perspective was recently provided in models of calcium
603 transient activity with GCaMP sensors in the dorsal striatum (Legaria et al., 2021). Given this,
604 one hypothesis consistent with our data is that vmPFC in ELS animals is lacking relevant
605 feedback on the efficacy of extinction learning, and this information may be provided via gamma
606 band oscillations.

607 This loss may be important for several reasons for interpreting our results. First, gamma
608 oscillations have been thought to reflect in part the activity of GABAergic interneurons (Buzsáki
609 and Wang, 2012; Cho et al., 2020; Sohal et al., 2009), and thus the ELS neurons displaying a
610 heightened excitability in this study may reflect the loss of this GABAergic regulation.
611 Compellingly, BLA afferents preferentially target PFC GABA interneurons during early
612 postnatal development (Cunningham et al., 2008), suggesting these pathways may be particularly
613 vulnerable to insult during early life. Furthermore, disruption of this pathways during early life
614 development appears to functionally alter and impair these arising BLA-PFC pathways, well-
615 characterized dysfunction of this pathway in ELS individuals and animal models (Fan et al.,
616 2014; Guadagno et al., 2018; Ishikawa et al., 2015; VanTieghem and Tottenham, 2018). Another
617 potential source of input may arise from the hippocampus, which has likewise been implicated in
618 fear-related changes in behavior in ELS-experienced animals (Reincke and Hanganu-Opatz,
619 2017). Consistent with this interpretation, high-gamma electrical stimulations in the fibria-fornix
620 preferentially enhanced coordination between PFC and hippocampus, suggesting a likely route of

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621 communication on this frequency (Helbing and Angenstein, 2020). Future investigations will
622 need to investigate these pathways, and in particular, why this frequency is uniquely disrupted
623 while others are relatively unaffected.

624 Finally, recent work has focused not only on responses to threats, but also how animals
625 come to learn about stimuli explicitly predictive of no-threat (i.e., safety). For our task, the CS-
626 cue served as a neutral stimulus without consequence, but also signaled the explicit absence of
627 any possibility of shock. This information about this safe cue appears to be reflected quite
628 differentially in the vmPFC of ELS and Control subjects. In general, vmPFC neurons in Controls
629 did surprisingly worse at discriminating between the CS+ and CS- than in ELS animals. While
630 both ELS and Controls were adequate at discriminating between CS+ and CS- stimuli, this was
631 not the case in inhibitory responses. Controls only showed discrimination between CS+ and CS-
632 on the third day of fear extinction, while ELS animals showed robust and reliable discrimination
633 between the CS+ and CS- throughout all days of extinction. These findings suggest the
634 possibility that ELS animals may be more vigilant and ascribe a greater salience to potentially
635 threatening stimuli, while therefore also better able to ascribe safety to non-threatening cues in
636 the same context. This interpretation suggests that separate signals and neurons in the vmPFC
637 may participate in the detection and significance of threat cues and their extinction (excitatory
638 responses), while another participates in the learned safety of explicitly neutral stimuli
639 (inhibitory responses). In this sense, ELS neurons were relatively impaired relative to Controls in
640 excitatory signaling about threats and extinction, while they were relatively enhanced relative to
641 controls in inhibitory signaling about safety signals. This intriguing dichotomy suggests discrete
642 pathways that may coordinate complex responses to environments with ambiguous and
643 competing information.

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644 In conclusion, these data demonstrate that ELS is a potent modulator of brain networks
645 that are essential for mediating appropriate and adaptive responses to a host of cognitive tasks
646 including relief from fear, abstinence from drugs of abuse, and adequate assessment of risk in
647 decision making. ELS experience, particularly early in development while the PFC and its limbic
648 network are still in the process of developing functional connectivity, can have lasting effects on
649 stimulus processing and behavioral responses to motivational stimuli. These data present new
650 insights into how ELS-related dysfunction may contribute to the wide variety of mental health
651 disorders that are precipitated by ELS and contribute to risk factors for disorders like addiction
652 and PTSD.

653 **Limitations**

654 While this work presents new and potentially important discoveries, it nevertheless has
655 many limitations which constrain the interpretation of the results. First and foremost, the design
656 of the ELS experience was not designed a priori as an early life stress model. As noted, the
657 animals were originally destined for another project and due to new animal procedures in a new
658 vivarium, the stressors that were presented were due to an unfortunate accident with animal
659 water packs that produced cage flooding during a critical development period. We were unsure
660 whether this experience would alter the brains of the affected litters, and as such, we felt they
661 were not sufficiently neurotypical for use as normal controls. Rather than sacrifice those litters,
662 we opted to keep and observe into adulthood. We are aware that there are different and more
663 standard ELS models of limited bedding, fragmented maternal care and others which have more
664 extensive use in the field (Molet et al., 2014). We look forward to being able to use these more
665 established models in the future to bring our observations into better alignment with those
666 procedures.

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667 Another significant limitation of this study was the lack of use of cross-fostering in
668 maternal care. Cohort effects from individual litters (such as unique features of maternal care or
669 parental genetics) as distinct from the ELS experience itself could contribute to the differences
670 seen here. Furthermore, we did not cull the litters to a set size. For example, dams with larger
671 litters may need to expend more energy and therefore create more scarcity for the pups than
672 dams with smaller litters. As noted above, the experimental procedure was not designed with
673 these important consideration in mind, and future studies will need to account for these features.

674 A third limitation is the low number of male subjects that we were able to record from in
675 adulthood. As noted in the methods, most of the males in were accidentally sacrificed prior to
676 adulthood due to mislabeling, though the female subjects were left intact. As such, the
677 unbalanced number of animals biased towards female subjects were pooled with the small
678 number of male subjects for analysis. In future studies, better-powered populations of males and
679 females would be valuable to tease apart ELS contributions to male and female development and
680 fear learning.

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881 Figure Legends
882
883 **Figure 1. A.** On PND6, weights of the ELS rats were lower than the unstressed Controls. **B.** In
884 adulthood (PND180), ELS subjects showed less time (*left*) and fewer initiated contacts with a
885 novel conspecific juvenile rat (*right*) in a JSI assessment. **C.** During Instrumental acquisition,
886 rats in the ELS group showed similar levels of motivation to press as controls across decreasing
887 schedules of reinforcement. **D.** In a conditioned suppression task, the fear-associated CS+ cue
888 suppressed lever pressing for food (VI60) more in ELS rats than Controls across three days of
889 fear extinction. **p*<0.05, main effect, ELS vs Control; #*p*<0.06, main effect, ELS vs Control;
890 &*p*<0.05, ELS vs Control CS+ on that Day.
891
892 **Figure 2.** Placements of array wires in the PFC. Controls (black/gray circles) and ELS animals
893 (red/orange circles) are shown primarily in the infralimbic cortex, with some wires extending
894 ventrally into the medial orbital and dorsal peduncular cortex, and some dorsally into prelimbic
895 cortex.
896
897 **Figure 3.** Heat plot representation of the population of recorded neural activity in the IL in
898 Controls (**A**) and ELS subjects (**B**) relative to the onset of the CS+ cue in the Conditioned
899 Suppression task. Color reflects magnitude of z-normalized firing with lighter colors indicating
900 greater firing rates (z > 1), while darker colors indicate inhibitory activity (z < 1). Cells on the plot
901 were sorted by the magnitude of the average firing rate during the first 1000ms after cue onset.
902 Brackets on the left of each plot indicate the range of cells for which the phasic response was at
903 least +0.5z above baseline ("excitatory"; *black top bracket*) or at least -0.5z below baseline
904 ("inhibitory"; *gray bottom bracket*). Bar to the right of each heatplot indicates the scale to translate
905 z score (from +4 to -4z) for each plot. **C.** Relative proportion of excitatory (EXC; greater than
906 +0.5z), inhibitory (INH; less than -0.5z), and non-phasic units relative to the first 1sec of cue onset.
907 ELS animals showed a significant increase in the proportion of EXC cells relative to Controls, χ^2_1
908 = 10.96, p = 0.0009
909
910 **Figure 4.** Phasic responses of vmPFC neurons to cue onset over repeated sessions of Conditioned
911 Suppression. Identified excitatory (**A**-**B**) and inhibitory (**C**-**D**) units were analyzed separately. **A.**
912 Both ELS (warm colors) and Control subjects (gray colors) showed rapid phasic responses to
913 presentations of the fear-associated CS+ that typically lasted less than 1sec following cue onset.
914 **B**. The average firing rate during the first 1sec following cue onset for each EXC cell for the CS+
915 (solid line) and the CS- (dashed line). C-D. Same as for A-B, but for maximum inhibitions (lowest
916 firing point). **p*<0.05, Control vs ELS (CS+);‡*p*<0.05, CS+ vs CS- (Controls); ᵝ*p*<0.05, CS+ vs
917 CS- (ELS).
918
919 **Figure 5.** Perievent spectrograms generated for each of the frequencies identified in each title.
920 Data are z-normalized by the average power in the baseline for each subject. At left in each
921 subfigure is the mean response in 200ms bins over the duration of the CS+ cue presentations
922 (Control: *black*; ELS: *red*). Vertical dotted line indicate cue onset and offset respectively. At right
923 in each subfigure is the average (excluding the first 400ms, which may reflect a non-associative
924 artifact). At left in black/gray are controls, and at right in red/pink are the ELS averages. In each
925 pair, the darker/left bar is the CS+, while the lighter/right bar is the CS-. *p<0.05, Control v ELS;
926 $^{\$}$p<0.05, Baseline period vs Cue period; $^{\&}$p<0.05, CS+ vs CS-.

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927 Figure 1.
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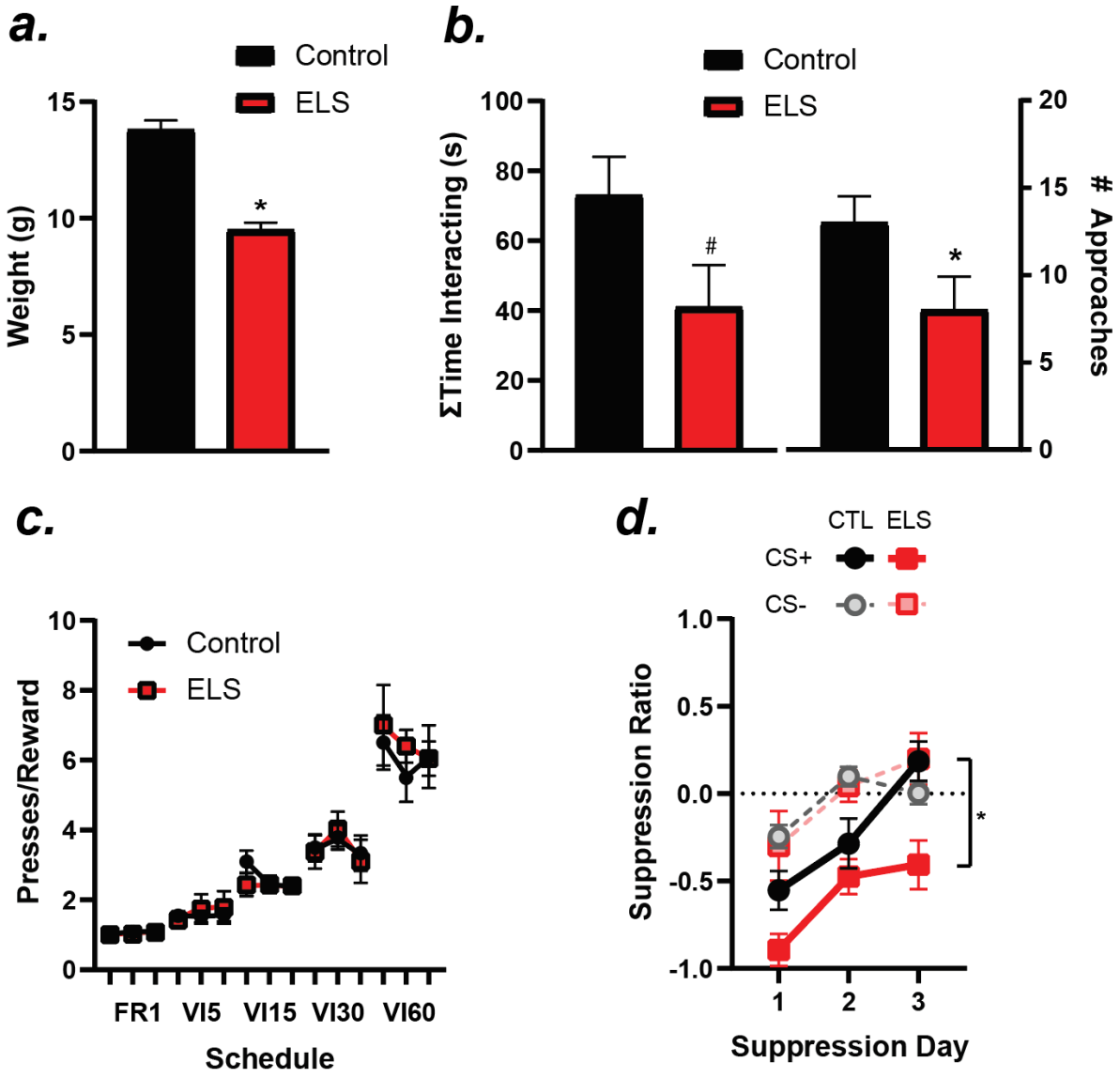

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931 Figure 2
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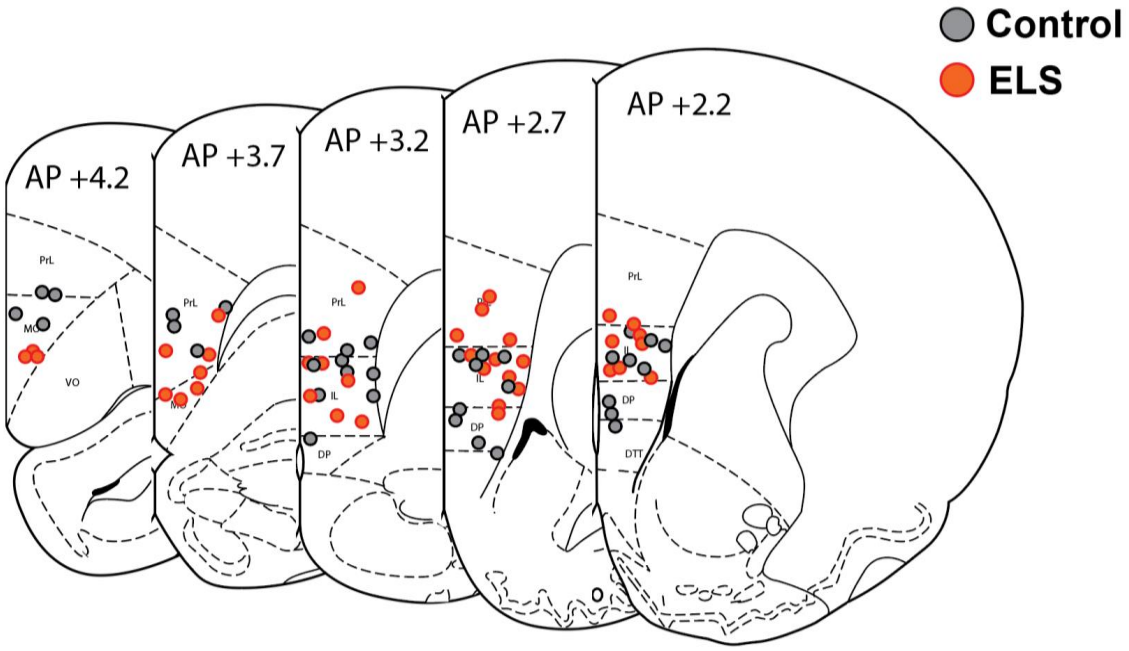

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936 Figure 3.
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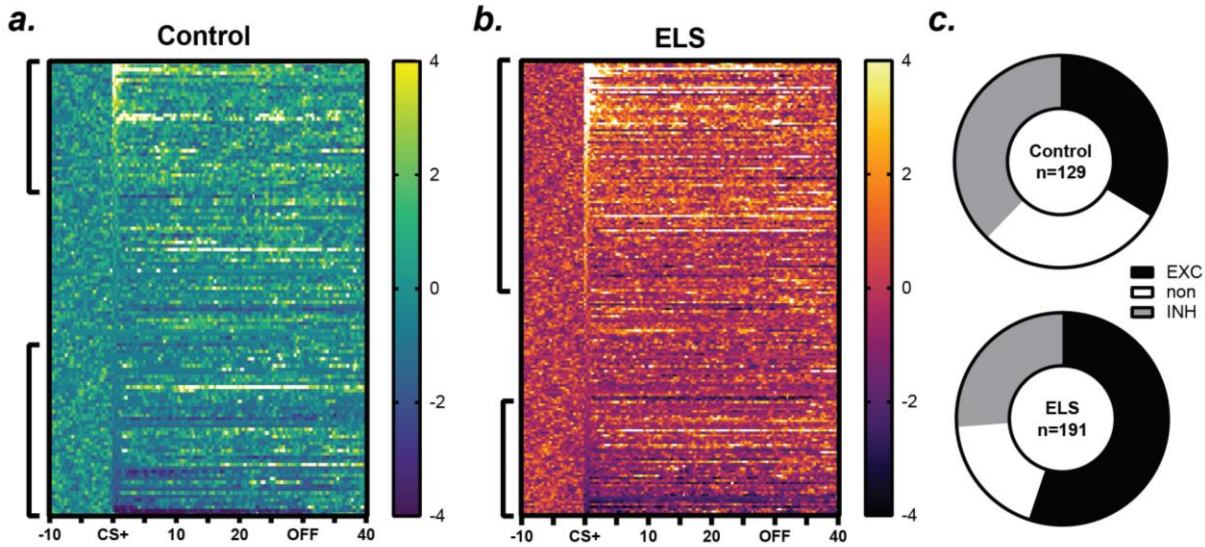

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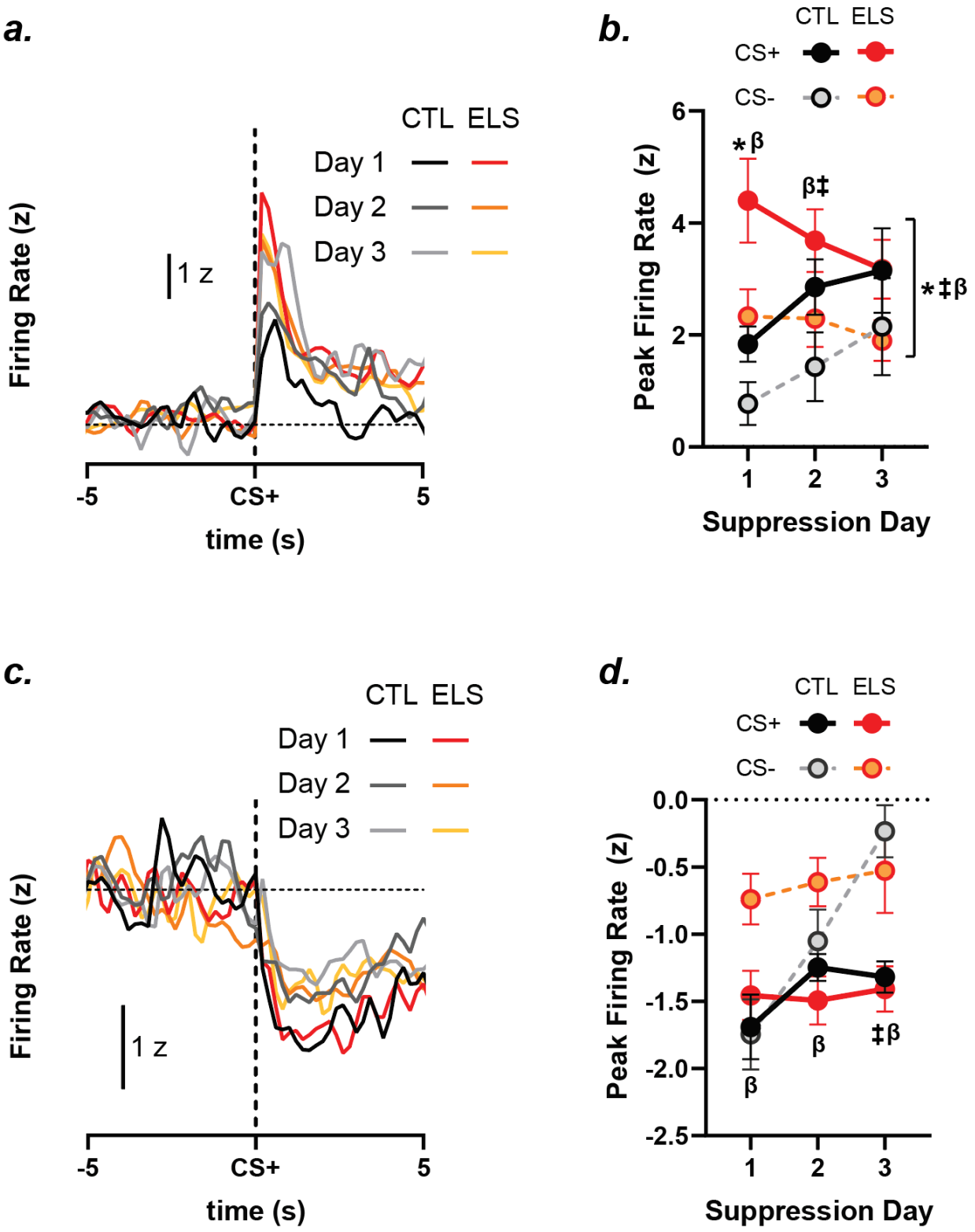
940 Figure 4.



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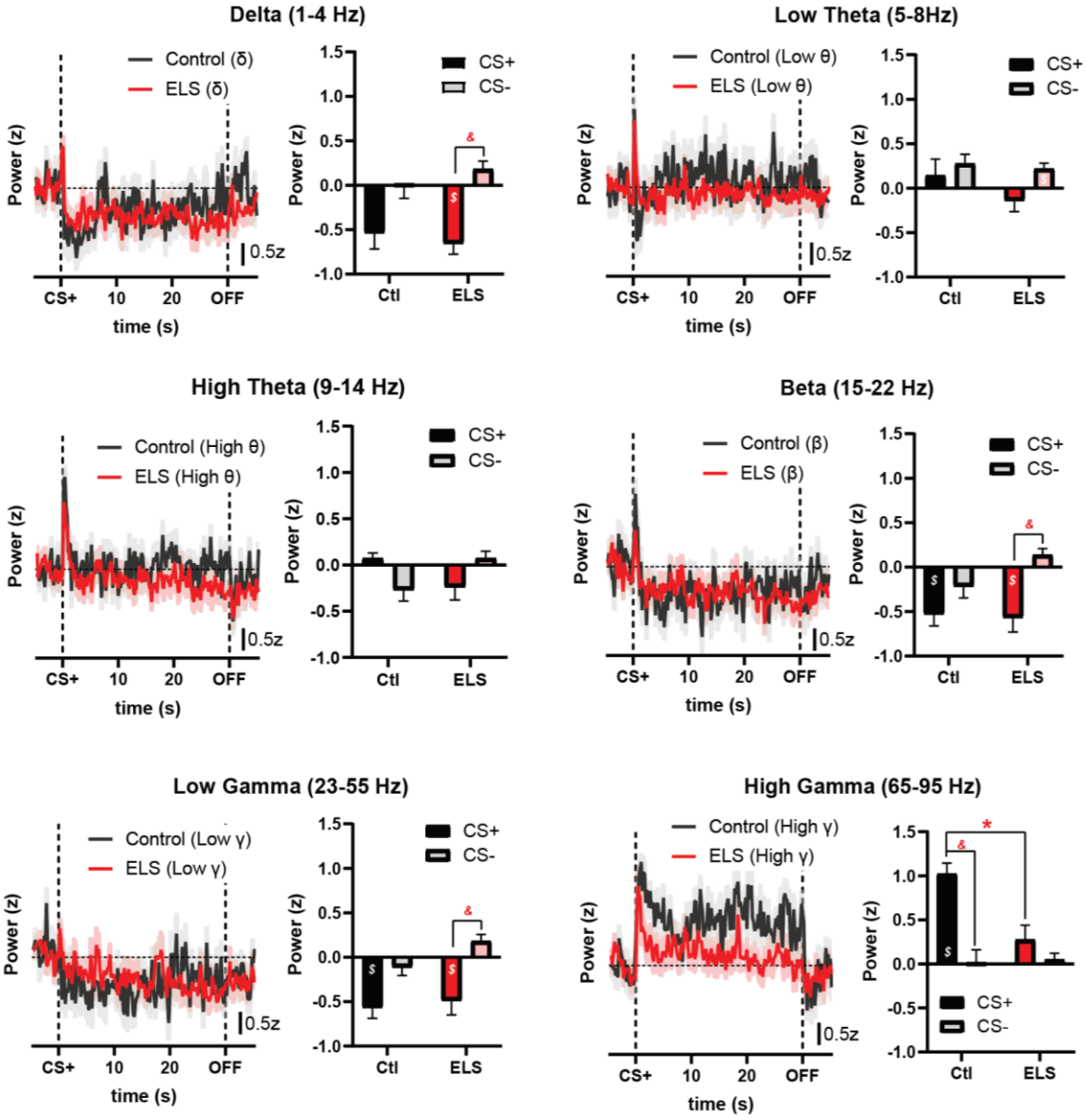
943 Figure 5.



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