Early life sleep disruption alters glutamate and dendritic spines in prefrontal cortex and impairs cognitive flexibility in prairie voles

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Abstract

Early life experiences are crucial for proper organization of excitatory synapses within the brain, with outsized effects on late-maturing, experience-dependent regions such as the medial prefrontal cortex (mPFC). Previous work in our lab showed that early life sleep disruption (ELSD) from postnatal days 14–21 in the highly social prairie vole results in long lasting impairments in social behavior. Here, we further hypothesized that ELSD alters glutamatergic synapses in mPFC, thereby affecting cognitive flexibility, an mPFC-dependent behavior. ELSD caused impaired cued fear extinction (indicating cognitive inflexibility), increased dendritic spine density, and decreased glutamate immunogold-labeling in vesicular glutamate transporter 1 (vGLUT1)-labeled presynaptic nerve terminals within mPFC. Our results have profound implications for neurodevelopmental disorders in humans such as autism spectrum disorder that also show poor sleep, impaired social behavior, cognitive inflexibility, as well as altered dendritic spine density and glutamate changes in mPFC, and imply that poor sleep may cause these changes.

1. Introduction

Early in development, neural synapses undergo morphological and functional changes necessary for the expression of species-typical adult behavior. Considerable research suggests synaptic reorganization, including synapse formation, growth, and pruning, is differentially shaped by rapid eye movement (REM), non-REM (NREM), and awake states (de Vivo et al., 2017; Diering et al., 2017; Vyazovskiy et al., 2008). These processes are especially sensitive to perturbations in sleep early in development (Li et al., 2017; Maret et al., 2011; Yang and Gan, 2012) when sleep amounts are highest (Roffwarg et al., 1966). In both humans and rodents, REM sleep time is especially high early in life comprising nearly 50% of all sleep. An age-related reduction in REM, typically occurring at ~2 years old in humans (Roffwarg et al., 1966) and approximately 14–21 days old in rodents (Cui et al., 2019; Frank and Heller, 1997; Rensing et al., 2018) may indicate a shift in the function of REM sleep from synaptic reorganization to neural repair that is conserved across species (Cao et al., 2020). Glutamate is differentially released within different brain regions across the various sleep-wake states (John et al., 2008; Saper and Fuller, 2017), and also plays a critical role in the stabilization and maturation of excitatory synapses (Cruz-Martin et al., 2012). Excitatory neurotransmission in mammals is driven, in part, by presynaptic vesicular glutamate release. This release is regulated by vesicular glutamate transporters, primarily vGLUT1 and vGLUT2, which are expressed in non-overlapping populations of presynaptic nerve terminals in the mammalian cortex (Fremeau et al., 2001). These terminals primarily make an asymmetrical synaptic contact onto dendritic spines, and spine morphology and density can inform how excitatory signals are processed (Häussler et al., 2000; Lefebvre et al., 2015). Accordingly, neurodevelopmental disorders commonly feature abnormalities in glutamate neurotransmission and related glutamatergic structures (e.g., dendritic spines, vGLUT1/2, and other excitatory synaptic markers) (Horder et al., 2013; Hutslar and Zhang, 2010; Purcell et al., 2001) along with altered sleep patterns compared to typically developing children (Buckley et al., 2010).

Higher order social learning, executive function, and cognitive flexibility in humans and rodents depends on the functioning of the prefrontal...
cortex (PFC) (see Dalley et al., 2004; Nelson and Guyer, 2011) for review. Subregions of the rodent PFC govern tasks requiring cognitive flexibility and rule change, including the extinction of emotionally salient associations (Laurent and Westbrook, 2009; Milad and Quirk, 2002; Morgan et al., 1993; Sierra-Mercado et al., 2011; Yuen et al., 2009) such as in cued fear. In both humans and rodents, the PFC is a late-maturing region with an extended sensitive period to experiences and environmental results. Accordingly, the PFC is often affected in neurodevelopmental disorders such as autism spectrum disorder (ASD), leading to characteristic impairments in social behavior and cognitive flexibility (Memari et al., 2013; Ozonoff et al., 2004).

Our recent research using the highly social prairie vole has shown that early life sleep disruption (ELSD), using a method that substantially reduces REM sleep but also increases wakefulness and fragments NREM sleep (Jones et al., 2019; Li et al., 2014; Sinton et al., 2009), results in long-term impairments in social behavior (Jones et al., 2019). Notably, using this method, there are no changes in measures of stress such as body mass, serum corticosterone, parental care, or anxiety-like behavior (Jones et al., 2019, 2020; Li et al., 2014). Here, we hypothesized that ELSD specifically affects glutamatergic neurotransmission in the late-maturing mPFC. In order to test this hypothesis, we examined effects of ELSD on 1) cognitive flexibility through cued fear extinction, a mPFC-dependent behavior, and 2) glutamatergic synaptic structure within the mPFC using a combination of light microscopic quantification of dendritic spines and ultrastructural immunohistochemical analysis using electron microscopy.

2. Materials and methods

2.1. Experimental design

Three experiments were run in separate cohorts of animals to evaluate behavioral and glutamatergic features in the adult prairie vole medial prefrontal cortex (mPFC) after either early life sleep disruption (ELSD) or control conditions early in life. In Experiment 1, cognitive flexibility to an emotional stimulus was quantified with cued fear conditioning followed by cued extinction. In Experiment 2, apical dendritic spine morphology of pyramidal neurons was quantified using Rapid Golgi staining in the prelimbic (PL) and infralimbic (IL) regions of the mPFC. In Experiment 3, ultrastructural features including glutamate density and size of vesicular glutamate transporter 1 (vGLUT1) and vesicular glutamate transporter 2 (vGLUT2) labeled presynaptic terminals and corresponding post-synaptic spines were quantified with electron microscopy in Layer 2/3 of the prelimbic cortex. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Portland VA Medical Center and were conducted in accordance with guidelines set forth by the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2. Subjects

Subjects were male and female adult prairie voles housed in clear polycarbonate cages (27 cm x 27 cm x 13 cm) in 14:10 light:dark cycle with lights on at 0700 h. Subjects were bred at our colony at the Portland VA, sourced from 48 litters from 22 breeder pairs and reared by both parents. The prairie vole colony originated from a colony at Emory University derived from field-caught prairie voles in Illinois. Colony diversity was maintained through generous bi-annual donations from researchers across the United States, most recently Dr. Lisa McGraw at North Carolina State University in 2014, Dr. Karen Bales at University of California, Davis in 2015, Dr. Zoe Donaldson at University of Colorado Boulder in 2017, and Dr. Zuxin Wang at Florida State University in 2019. Breeder pairs were checked each morning at lights on for the presence of pups and the day of pup discovery was designated as postnatal day (P1). Voles were weaned at P21 and socially housed with same sex littermates (2–4/cage). Female prairie voles were rehoused to a female-only colony room at this time, as they are male-induced ovulators, and are presumed to have been in anestras for the duration of this study. Prairie voles had ad libitum access to water and a mixed diet of rabbit chow (LabDiet Hi-Fiber Rabbit), corn (Nutrena Cleaned Grains), and cracked oats (Grainland Select Grains). Cotton nestlets and a wooden block or stick for chewing enrichment were added to each cage and replaced weekly with cage change.

2.3. ELSD paradigm

Prairie vole litters in their home cages were placed on a standard laboratory orbital shaker for one week, with both parents, from postnatal days 14–21 (ELSD group). The orbital shaker was programmed with an automatic timer to gently agitate the cage at continuous intervals at 110 RPM (10 s every 110 s; See (Jones et al., 2019)). Modifications to standard housing consisted of replacing water bottles with hydrogel to prevent leaking and cage cards were secured to prevent excessive auditory disturbance. The Control group consisted of litters transferred to the same colony room containing the shakers from P14-21 but were not physically sleep disrupted. Control cages had hydrogel provided but retained their water bottles as an additional source of hydration. We have previously found that this method of early life sleep disruption does not increase serum corticosterone levels in pups and does not alter parental care (Jones et al., 2019).

2.4. Experiment 1 – cued fear conditioning and extinction

2.4.1. Experiment 1 design

Adult prairie voles (P75–P90) that had undergone either ELSD (n = 14 male; n = 8 female) or control (n = 8 male; n = 10 female) early life sleep conditions underwent a four-day cued fear conditioning and extinction paradigm. Voles underwent cued fear conditioning following by two days of extinction and finishing with one day of long-term memory tests for extinction retention. Each procedure was separated by 24 h and conducted in the second half of the light cycle. Behavioral tests were conducted by the same experimenter each day (C.E.J.).

2.4.2. Apparatus and stimuli

All fear conditioning procedures took place in clear plexiglass chambers (40.8 cm x 14 cm x 18.4 cm) within black sound attenuating chambers fitted with fans, which provided background white noise at ~60 dB. Fear conditioning chambers were equipped with steel rod floors connected to a shock generator (Omnitech). Chambers were wiped with water and patted dry between each animal. The CS was a tone (3 kHz, 80 dB) 10 s in duration, and the US was a 0.7 mA foot-shock, 1 s in duration.

2.4.3. Behavioral testing procedures

Fear conditioning (day 1) – after a 6 min habituation to the chamber each vole received 5 CS presentations (fixed inter-trial interval = 60 s), each co-terminating with the US. After fear conditioning, voles were immediately removed from the chambers and returned to their home cage. All cage mates were fear conditioned according to the same parameters and remained socially housed throughout the duration of the experiment.

Extinction 1 and 2 (days 2 and 3) – after a 6 min habituation, each vole received 19 CS presentations in the absence of the US (fixed ITI = 60 s)
in the same context as fear conditioning. Voles underwent two extinction sessions separated by 24 h.

Long-term memory (day 4) – approximately half of the voles (n = 9 Control and n = 9 ELSD) underwent long-term memory tests on day 4 to test for extinction retention. Long-term memory tests consisted of 3 CS presentations in the absence of the US (fixed ITI = 60 s). The other half were not tested on this day due to equipment failure.

2.4.4. Scoring and analysis
A trained experimenter (C.E.J.) blind to both sex and early life sleep condition scored total time freezing post-hoc with a digital stopwatch for the duration of each CS. Freezing was defined as the absence of any movement, excluding breathing and whisker twitches. The total time spent freezing throughout CS presentation was expressed as a percentage of CS duration (10 s). Approximately 30% of sessions were also scored by a second scorer (R.J.O) and inter-rater reliability was high (r = 0.897). Freezing was analyzed with repeated measures ANOVA (within subjects factor = CS number; between subjects factors = sleep group and sex). Extinction data did not meet requirements for sphericity and p values were Greenhouse-Geisser corrected.

2.5. Experiment 2 – spine morphology and density: Rapid Golgi method

2.5.1. Tissue collection and processing
Voles underwent either ELSD or control conditions from P14–P21. As adults (P77–P80) animals were euthanized by an overdose of isoflurane followed by decapitation and brains were rapidly removed (P.T.W., C.E. J.). Brain tissue was fixed and stained in accordance with manufacturer’s instructions (Rapid GolgiStain™ Kit, FD Neurotechnologies, Inc.). In brief, brains were bisected into hemispheres, impregnated in Golgi solution and kept in the dark for two weeks, and then kept in a sucrose solution for three days prior to sectioning. Brains were sectioned at 200 μm (Precisionary Instruments, Greenville, NC), mounted on gelatin coated slides, dehydrated in ethanol series (4 min each: 50% (x1), 75% (x1), 95% (x1), 100% (x4)), cleared with xylenes (three times, 4 min each rinse), and cover slipped with permount. Data presented here were collected from the right hemisphere. Euthanasia was performed between 1000 h and 1200 h (ZT3-ZT5) for all animals.

2.5.2. Quantitative analyses of spine density and spine morphology
To quantify dendritic spine morphology, Golgi-stained, pyramidal neurons were imaged using a 40x objective (NA = 0.85) on a Leica microscope. Z-stack images (80 μm total on the Z-axis; optical section thickness = 2 μm; 41 images per stack; image size, 2048 × 1535 pixels (0.1774 × 0.1774 × 2 μm) were acquired using a DFC36 FX camera. One experimenter acquired all images (A.Q.C.). Each image stack was extracted using ImageJ software (NIH, Bethesda, MD) and subsequently imported into RECONSTRUCT software (Fiala, 2005) for analysis as described in (Moore et al., 2020). This method allows for dendrites and spines to be measured in the x, y, and z planes. Pyramidal neurons were imaged from cortical layers 2/3 and 5 in the PL and IL cortices. The IL and PL were distinguished using the atlas of Frank and Heller (1997) as well as defining cytoarchitecture of Layer 2 in the IL compared to the PL. Only fully impregnated neurons (e.g. no sudden breaks in branches) clearly distinguishable from neighboring neurons were selected for quantification. For each animal, four segments (10–20 μm) were imaged from apical oblique dendrites from 2-4 pyramidal neurons and values averaged together to create a representative sample from each animal for statistical analysis. To control for sources of variability, segments were selected from each brain to include one secondary and one tertiary dendritic branch from segments both proximal (<80 μm) and distal (>90 μm) to the soma wherever staining permitted (4 segments total for each brain). Spine density, length, and width were quantified, and in this sample, were not significantly different between secondary and tertiary branches or proximal and distal locations and values were averaged together for one representative value for analysis from each animal. All spine quantification was conducted by one experimenter (A.Q.C.) with a small subset confirmed by a second experimenter (R.J.O.), both blind to sex and group. The spine density of each segment was calculated by dividing the total number of spines by the length of the corresponding segment. In addition, spine length (L) was measured from the base of the dendritic spine to the tip and spine head width (W) was measured at the widest point visible. Values met criteria for parametric testing and were compared using ANOVA (between subjects factors = sleep group and sex) for each layer (2/3 or 5) and P values required for significance were Bonferroni corrected to account for multiple comparisons (alpha = 0.025).

2.6. Experiment 3 – electron microscopy

2.6.1. Experiment 3 design
Prairie voles underwent either ELSD (n = 4 female; n = 6 male) or Control conditions (n = 3 female; n = 4 male) from P14–P21 and as adults (P70–P110) were deeply anesthetized with ketamine/xylazine cocktail (0.2 mL i. p.) and transcardially perfused with 6 mLs of phosphate buffer (0.1 M, pH 7.3), containing 1000 units/ml of heparin, then followed immediately by 50 mLs of EM fixative (2.5% glutaraldehyde/0.5% paraformaldehyde/0.1% picric acid in 0.1 M phosphate buffer, pH 7.3), as previously described (Moore et al., 2020) (C.K.M). Brains were collected in phosphate buffer, cut in half coronally at the level of the hypothalamus and then further processed in EM fixative in the Biowave (Pelco BioWave, Ted Pella, Inc.) as recently detailed (Moore et al., 2020). Brains were collected for EM during the light portion of the light cycle between ZT3-6.

2.6.2. Electron microscopy (EM) immunolabeling
Slices containing the prefrontal cortex were processed for EM and imaged as previously described (Moore et al., 2020; Parievsky et al., 2017). The tissue was processed using antibodies against the vesicular glutamate transporter 1 (vGLUT1: Synaptic Systems, 1:1000, rabbit polyclonal, #135303) and vesicular glutamate transporter 2 (vGLUT2: Synaptic Systems 1:100, rabbit polyclonal, #135403), using our standard immunohistochemistry (IHC) protocol (Parievsky et al., 2017; Spinelli et al., 2014; Walker et al., 2012). Using tissue samples from each treatment group (3–4 slices/animal), the vGLUT1 and vGLUT2 antibodies were run with all processing carried out in a microwave oven (Pelco BioWave, Ted Pella, Inc.) on the same day. During the IHC processing for electron microscopy Triton X-100 was not used in order to preserve the tissue morphology. Tissue was prepared for electron microscopy as previously reported (Moore et al., 2020; Parievsky et al., 2017; Spinelli et al., 2014; Walker et al., 2012).

2.6.3. EM embedding
Tissue was then processed using the same Biowave tissue processor as noted above, with the temperature restricted to less than 60 °C for all steps. Tissue was exposed to a solution of 1% osmium tetroxide (OsO₄) (Electron Microscopy Sciences, Hatfield, PA) in 1.5% potassium ferricyanide (Electron Microscopy Sciences, Hatfield, PA) at 100 W, with the vacuum cycling for 13 min (cycling the magnetron for 3 min on/2 min off/3 min on/2 min off/3 min on). The tissue was then rinsed in deionized Millipore-filtered H₂O (Di H₂O), and the OsO₄ was removed.
gold labeling within the mitochondria (i.e., metabolic pool) was synaptic vesicle membrane (i.e., vesicular pool), the number located of glutamate labeling within the nerve terminals, the number of magnification of neuropil (an area containing the highest numbers of synapses) at a final making an asymmetrical synaptic contact onto a spine throughout the (JEOL) electron microscope, photographs were randomly taken in DAB labeling (Meshul et al., 1994; Moore et al., 2020). Using a JEM-1400 in no immuno-gold labeling, showing the specificity of the glutamate AB_2338016), tagged with 12 nm gold particles. We previously reported the tissue. The secondary antibody was goat anti-rabbit IgG (Jackson Co, St. Louis, MO, #G6642) and a secondary antibody tagged with 12 nm gold particles (goat anti-rabbit, 1:50: Jackson ImmunoResearch, West Grove, PA: AB 2338016) was carried out as previously described (Moore et al., 2020; Parievsky et al., 2017). The primary glutamate antibody, as previously characterized (Moore et al., 2020; Phend et al., 1992), was diluted in tris buffered saline with Triton X-100 (TBST, pH 7.6) in blocking solution [0.5% bovine serum albumin (BSA)] (Electron Microscopy Sciences, Hatfield, PA). Aspartate (1 mM) was added to the glutamate antibody mixture 24 h prior to incubation with the thin-sectioned tissue to prevent any cross-reactivity with aspartate within the tissue. The secondary antibody was goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA; diluted 1:20 in TBST pH 8.2, AB 2338016), tagged with 12 nm gold particles. We previously reported that incubation of the glutamate antibody with 3 mM glutamate resulted in no immuno-gold labeling, showing the specificity of the glutamate labeling (Meshul et al., 1994; Moore et al., 2020). Using a JEM-1400 (JEOL) electron microscope, photographs were randomly taken in DAB labeled areas of Layer 2 (at the leading edge of the tissue section). This layer was easily identifiable within the cortex since this region is just below Layer I, which contains few, if any, neuronal cell bodies.

2.6.4. Morphological analysis

Photographs were taken of DAB labeled terminals (vGlut1, vGlut2) making an asymmetrical synaptic contact onto a spine throughout the neuropil (an area containing the highest numbers of synapses) at a final magnification of × 46,200 by an individual blinded to the experimental groups, using a digital camera (AMT, Danvers, MA). For quantification of glutamate labeling within the nerve terminals, the number of immuno-gold particles located either within, or at least touching the synaptic vesicle membrane (i.e., vesicular pool), the number located outside the synaptic vesicles (i.e., the cytoplasmic pool), and those associated with mitochondria, were counted. The density of glutamate gold labeling within the mitochondria (i.e., metabolic pool) was excluded from the synaptic vesicle/cytoplasmic pool analysis, as previously reported (Meshul et al., 1994; Moore et al., 2020). There are no mitochondria located within dendritic spines. The vesicular and cytoplasmic pools were combined since the cytoplasmic pool is very small (<10%) compared to the vesicular pool (Meshul et al., 1999). We have reported that nerve terminals making a symmetrical contact contain GABA (Meshul et al., 1999), the precursor for which is glutamate. Therefore, nerve terminals making a symmetrical contact will naturally contain some glutamate immunolabeling and cannot be considered immuno-negative as a way of determining a ratio between glutamatergic and GABAergic terminals (Meshul et al., 1994, 1999). The metabolic pool is also relatively small and thus unlikely to be a major source of variation in labeling density. The density of gold particles/µm² of nerve terminal area for the vesicular/cytoplasmic pool was determined for each animal and the mean density for each treatment group calculated. Background labeling was determined within glial cell processes and was found to be about 10 immuno-gold-labeled particles/µm² (Meshul et al., 1994, 1999). This was subtracted from the density of presynaptic immuno-gold-labeled glutamate within the vGlut1+ or vGlut2+ nerve terminals. The area of the vGlut1 and vGlut2 labeled terminals was measured along with counting the number of gold particles in the terminal area to determine the density of presynaptic glutamate immuno-gold labeling. The post-synaptic structure was noted as to the type (spine). Photographic analysis was carried out using ImagePro Premier (MediaCybernetics), and statistical analysis was performed with JMP 11 (SAS). The number of gold particles per terminal, the area, and the density (# gold particles/µm²) was determined for the nerve terminal. Only presynaptic terminals contacting spines were used for analysis.

Independent sample t-tests (two tailed) were conducted on average glutamate density in presynaptic terminals labeled with either vGlut1 or vGLUT2 and alpha values were Bonferroni corrected to account for multiple comparisons. With this correction, a p-value < 0.025 was required to reject the null hypothesis. Outliers were excluded from analysis and were defined as values greater than 2 standard deviations above or below the mean. Males and females were combined for analysis.

3. Results

All experiments were conducted in adult prairie voles that underwent either an Early Life Sleep Disruption (ELSD) paradigm or Control conditions continuously from postnatal days (P) 14–21 (Jones et al., 2019, 2020). Three experiments were conducted sequentially 1) behavioral testing for cued fear conditioning and extinction, 2) dendritic spine quantification and morphology using Rapid Golgi staining of pyramidal neurons in both superficial and deep layers of the mPFC including both infra- and pre-limbic cortices, and 3) electron microscopy of vGlut1 and vGlut2 labeled synapses. Separate animals were used in each experiment.

3.1. ELSD impairs extinction acquisition after cued fear conditioning

In Experiment 1, cognitive flexibility was measured by first exposing voles to cued fear conditioning (tone cue paired with a mild footshock) followed by two days of extinction (19 non-reinforced cue presentations per session; Fig. 1a for experimental timeline). Cognitive flexibility was inferred when voles showed decreased freezing behavior, an expression of fear, during the extinction sessions after exposing voles to cued fear conditioning (tone cue paired with a mild footshock) followed by two days of extinction (19 non-reinforced cue presentations per session; Fig. 1a for experimental timeline). Cognitive flexibility was inferred when voles showed decreased freezing behavior, an expression of fear, during the extinction sessions after first learning to display a fear response to the cue. Males (ELSD n = 13; Control n = 10) and females (ELSD n = 8; Control n = 10) are combined for visual representation (Fig. 1).

Both groups acquired conditioned freezing to the CS during fear acquisition and there were neither group (repeated measures ANOVA, effect of group: F (4,148) = 1.27, p = 0.267) nor sex (effect of sex: F (4,148) = 1.02, p = 0.319) differences (Fig. 1b). On the first extinction day (Fig. 1c), there was a significant within subjects effect of
ANOVA, within subjects factor CS number: the beginning of extinction 1.
e) Long-term memory tests of extinction indicate that both groups retain their levels of extinction but ELSD voles froze more than Controls. Data are mean ± SEM. Asterisks indicate significant between group effects. *p < 0.05, **p < 0.01. # indicates significant group x CS number interaction p < 0.05. US = unconditioned stimulus; CS = conditioned stimulus.

conditioned stimulus (CS) number on freezing levels (repeated measures ANOVA, within subjects factor CS number; F (18,660) = 20.584, p < 0.0001) as well as a significant interaction between freezing over the first extinction period and early life sleep group (repeated measures ANOVA, CS number x sleep group interaction; F (18,684) = 2.677, p = 0.005). During the second day of extinction (Fig. 1d) there was also a significant reduction of freezing over CS number (repeated measures ANOVA, between group effect of CS number; F (18,630) = 13.583, p < 0.0001). This rate of reduction was no longer influenced by the ELSD group (repeated measures ANOVA, CS number x sleep group interaction; F (18,630) = 0.906, p = 0.528), nor was it influenced by sex (repeated measures ANOVA, CS number x sex interaction; F (18,630) = 0.923, p = 0.512). Overall, ELSD voles froze more than Controls during both extinction sessions (Extinction 1: effect of sleep group; F (1,37) = 6.981, p = 0.012; Extinction 2: effect of sleep group; F (1,35) = 24.985, p < 0.0001) and there were no significant effects of sex (Extinction 1: effect of sex; F (1,37) = 0.028, p = 0.868; Extinction 2: effect of sex; F (1,35) = 2.845, p = 0.101) or interactions (Extinction 1: sleep group x sex interaction F (1,37) = 0.093, p = 0.763; Extinction 2: sleep group x sex interaction; F (1,35) = 2.448, p = 0.127). These results suggest that ELSD impairs extinction learning after cued fear conditioning and may be an indicator of reduced cognitive flexibility compared to Controls.

A subset of animals (n = 18) were tested 24 h later for long-term extinction retention with three CS presentations (Fig. 1e). ELSD voles froze significantly more than Controls over the three cues (repeated measures ANOVA, between group effect of sleep group; F (1,14) = 22.448, p < 0.001) which was driven by increased freezing in the males (repeated measures ANOVA, sleep group x sex interaction; F (1,14) = 5.077, p = 0.041). There was not a main effect of sex on freezing during the long-term memory test (repeated measures ANOVA, between group effect of sex; F (1,14) = 1.175, p = 0.297).

3.2. ELSD increases spine density in PL Layers 2/3 – Rapid Golgi

Based on the fear extinction behavior, our a priori hypothesis was that the mPFC would show changes in dendritic spine density after ELSD, as assessed by Rapid Golgi staining. Within the rodent PFC, tasks requiring cognitive flexibility and rule change depend on functioning of the medial subregions, with opposing roles of the more dorsally located prelimbic region (PL) and the more ventrally located infralimbic region (IL) in activation and inhibition of fear responses, respectively (Sierra-Mercado et al., 2011). Based on this literature, we further hypothesized that PL and IL would show unique responses to ELSD due to their opposing roles in extinction learning. Separate ANOVAs were used to analyze data for Layers 2/3 and Layer 5 and alpha values were Bonferroni corrected to account for multiple comparisons (with correction, p < 0.025 is needed to reject the null hypothesis).

In Layers 2/3 of PL, there were significantly more spines per μm in ELSD animals (n = 7 males; n = 6 females) compared to Controls (n = 8 males; n = 4 females) (ANOVA, main effect of sleep group; F (1,21) = 24.614, p < 0.0001) and no significant effect of sex (ANOVA, main effect of sex; F (1,21) = 2.087, p = 0.163; sleep group x sex interaction; F (1,21) = 1.127, p = 0.301) (Fig. 2a, left panel). In Layer 2/3 of IL, ELSD did not affect spine density (ANOVA, main effect of sleep group; F (1,15) = 0.858, p = 0.369) but there was a trend towards a main effect of sex, with increased spine density in female voles (ANOVA, main effect of sex; F (1,15) = 4.323, p = 0.055; group x sex interaction; F (1,15) = 4.734, p = 0.046) that did not meet significance requirements with post-hoc corrections for multiple cortical layers (Fig. 2a, right panel) (n = 5 Control males; n = 4 Control females; n = 5 ELSD males; n = 5 ELSD females). There were no significant differences in spine density in Layer 5 of PL (ANOVA, main effect of sleep group; F (1,12) = 0.231, p = 0.639, main effect of sex; F (1,12) = 3.378, p = 0.091; group x sex interaction; F (1,12) = 0.147, p = 0.708) (Fig. 2a, right panel) (n = 6 Control males; n = 2 Control females; n = 5 ELSD males; n = 5 ELSD females), or IL cortices (ANOVA, main effect of sleep group; F (1,12) = 0.231, p = 0.639, main effect of sex; F (1,12) = 3.378, p = 0.091; group x sex interaction; F (1,12) = 0.147, p = 0.708) (Fig. 2a, right panel) (n = 2 Control males; n = 4 Control females; n = 5 ELSD males; n = 5 ELSD females).
3.3. ELSD causes thinner spines in PL Layers 2/3 – Rapid Golgi

Spine morphology was quantified as an indicator of maturity using the average length:width ratio (LWR) for spines in each segment and analyzed in an identical manner as spine density. In Layer 2/3 of PL, voles that underwent ELSD from P14-21 had higher LWR compared to controls indicative of longer and thinner spine morphology (Fig. 2b, left panel) (ANOVA, main effect of group: $F(1,21) = 20.520, p = 0.0002$) and females had higher LWR compared to males (ANOVA, main effect of sex: $F(1,21) = 18.564, p = 0.0003$; group × sex interaction; $F(1,21) = 3.862, p = 0.063$). LWR were also higher after ELSD in Layer 2/3 of IL (Fig. 2b, right panel) (ANOVA, main effect of sleep group: $F(1,15) = 7.379, p = 0.016$) and the effect of sex did not reach significance (ANOVA, main effect of sex: $F(1,15) = 3.254, p = 0.091$; group × sex interaction; $F(1,15) = 1.872, p = 0.191$). There was no effect of ELSD on LWR in Layer 5 of PL (Fig. 2b, left panel) (ANOVA, main effect of sleep group: $F(1,14) = 0.275, p = 0.608$) and no effect of sex (ANOVA, main effect of sex: $F(1,14) = 0.628, p = 0.441$; group × sex interaction; $F(1,14) = 1.970, p = 0.182$), nor in Layer 5 of IL (Fig. 2b, right panel) (ANOVA, main effect of group: $F(1,12) = 3.184, p = 0.100$) nor an effect of sex (ANOVA, main effect of sex: $F(1,15) = 1.576, p = 0.233$; group × sex interaction: $F(1,15) = 2.231, p = 0.161$). Combined, these results suggest that ELSD leads to both increased spine density in PL and longer, thinner spines in the same prefrontal brain region that underlies the expression of cued fear extinction.

3.4. ELSD decreases the density of glutamate immuno-gold labeling and size of vGLUT1-labeled presynaptic nerve terminals in PL Layers 2/3 – electron microscopy

Given our finding of increased spine density and changes in spine morphology (increased LWR) within PL Layers 2/3 after ELSD, we next focused specifically upon examination of ultrastructural changes within glutamatergic synapses of Layers 2/3 of PL. Sections were double-labeled with either glutamate-immunogold + vGLUT1 or glutamate-immunogold + vGLUT2. vGLUT1-labeled presynaptic nerve terminals are presumed to originate from cortico-cortical synaptic inputs into the mPFC, whereas vGLUT2-labeled presynaptic nerve terminals are presumed to originate from thalamic inputs into the mPFC (Fremeau et al., 2004; Kaneko et al., 2002).

In vGLUT1-labeled presynaptic nerve terminals, glutamate density (number glutamate immunogold particles/μm$^2$) was lower in ELSD voles ($n = 8$; 5 male and 3 female) compared to Controls ($n = 6$; 3 males and 3 females) (independent samples t-test, two tailed: $t(12) = 3.149; p = 0.008$). Glutamate density was not affected by early life sleep group.
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(ELSD n = 7 (3 males, 4 females); Controls (spines: n = 6: 3 males, 3 females; pre-synaptic area: n = 9: 6 males, 3 females) (independent samples t-test, two-tailed: t (11) = 2.605; p = 0.024; pre-synaptic area t (14) = 4.259, p = 0.001) (Fig. 3c and d). There was no difference in the average presynaptic terminal area of vGLUT2-labeled terminals (independent samples t-test, two tailed: t (11) = 1.793, p = 0.100) nor their corresponding spine areas (independent samples t-test, two tailed: t (11) = 0.369, p = 0.719) between ELSD (spines: n = 7, 4 males, 3 females; pre-synaptic area: n = 7, 3 males, 4 females) and Controls (n = 6, 4 males, 2 females).

4. Discussion

We have previously shown in the highly social prairie vole that early life sleep disruption (ELSD) during the third postnatal week of development, resulted in a profound impairment in social behavior later in life (Jones et al., 2018). Here, we show that adult voles that underwent ELSD also showed significantly impaired extinction of cued fear, a behavior that is dependent on mPFC development (Kim et al., 2009) and could be attributed to impaired cognitive flexibility. Furthermore, adult voles subjected to ELSD showed increased dendritic spine density and spine immaturity, as well as decreased glutamate labeling within vGLUT1+ nerve terminals, both indicative of altered glutamatergic neurotransmission within the prelimbic (PL) region of the mPFC. Our experimental results mirror human neurodevelopmental disorders including autism spectrum disorder that also show poor sleep (Buckley et al., 2010), impaired social behavior (for review see (Volkmar et al., 2004), cognitive inflexibility (for review see (Geurts et al., 2009)), as
well as altered dendritic spine density and glutamate changes in the brain (Hutsler and Zhang, 2010; Purcell et al., 2001). Taken together, these results have profound implications about the directionality and potential mechanisms underlying the relationship between early life sleep, neural plasticity, and behavioral development.

4.1. ELSD impairs cognitive flexibility

We found that ELSD voles did not extinguish a cued fear association to the same level as Controls, indicating impaired cognitive flexibility. In humans with ASD, cognitive and behavioral rigidity are measured in laboratory settings through deficits in reversal learning (Loveland et al., 2008; South et al., 2012) and extinction (Top et al., 2015). Accordingly, animal models of ASD also show similar impairments in reversal learning (e.g. Oxt−/− mouse line (Sala et al., 2011)) and fear extinction (e.g. VPA exposed rats (Banerjee et al., 2014; Markram et al., 2008)), supporting the use of this behavioral test as a measure of cognitive flexibility.

Furthermore, we found that ELSD voles showed higher levels of freezing behavior during extinction and long-term memory tests. Although ELSD voles did not extinguish as completely as Controls, there was no impairment in recalling the extinction memory that was formed after 24 h, a behavior that relies on the IL subregion of the mPFC (Laurent and Westbrook, 2005; Quirk et al., 2000) and was therefore not the focus of these studies. However, ELSD and Control voles froze at similar levels during both the early cues of extinction and during fear acquisition suggesting that the acquisition and long-term (24 h) retention of an auditory cued fear association is not affected by ELSD. Of note, there was no difference in the rate of extinction on the 2nd day of extinction learning between ELSD and Control voles. However, the floor levels of freezing on day 2 in Controls make further interpretation of extinction rate difficult. Extinction of cued fear is a mPFC-dependent task, a brain region of particular relevance to ELSD given its experience-dependent maturation that occurs throughout the early postnatal period. Cued fear extinction requires reversal and suppression of previously learned fear responses. At the neural level, cued fear extinction recruits a circuit involving the amygdala, hippocampus, and both the PL and IL subregions of mPFC. These mPFC regions are well documented in the literature for their opposing responses to fear learning and extinction: with the PL activating the fear response and the IL inhibiting fear responding via direct and indirect connections with the amygdala (Courtin et al., 2014; Quirk et al., 2000).

Our results suggest that ELSD may affect behavioral response selection when faced with an emotionally salient challenge, including a cue that previously signaled a footshock, or similarly, partner preference behavior (Jones et al., 2019).

4.2. ELSD increases immature spines in PL Layers 2/3

Given the dependence of extinction behavior on both PL and IL subregions of the mPFC, we next examined the morphology of dendritic spines on pyramidal neurons in both the PL and IL of adult ELSD or Control voles. We found that spine density in PL (but not IL) was increased in ELSD voles compared to Controls, and that this effect was specific to mPFC Layers 2/3. Furthermore, we found that spine length to width ratio was also increased in Layers 2/3 in ELSD voles compared to Controls, indicating longer/thinner spines and a potential shift towards immaturity (Holtmaat et al., 2005). These effects were not present in Layer 5.

In the rodent mPFC, PL is positioned to integrate both limbic and sensory information from the neocortex. While the rodent PL and IL both receive afferent projections from primarily limbic regions and the midline thalamus (Condé et al., 1990; Hoover and Vertes, 2007; Swanson, 1981; Van Eden et al., 1992), the main anatomical difference is the origin of their cortical inputs. Afferents to PL come from other areas of PFC, whereas afferents to IL come from PL and, more strongly, hippocampal CA1. Thus, PL contains strong cortico-cortical connections with other frontal regions responsible for integrating sensory, limbic, and timing information (Hoover and Vertes, 2007). The specificity of effects of ELSD on dendritic spine density within PL (and not IL) may indicate a developmental vulnerability for neocortical inputs.

The layers of the mammalian cortex reveal a topographic organization that informs function; pyramidal neurons in the superficial cortical layers have characteristic connectivity with other cortical regions. Within the rodent mPFC, thalamic afferents are most prominent in deeper layers (e.g., Layers 4 and 5), compared to more superficial layers (e.g. Layers 2/3), where afferents tend to be from adjacent PFC and other cortical regions (Hoover and Vertes, 2007). The fact that we observed changes in Layers 2/3 and not Layer 5 suggests that the cortical, rather than thalamic, afferents to mPFC are more sensitive to ELSD during this particular developmental window of P14-21.

Spine development in the human PFC also occurs postnatally during an extended developmental window within the superficial cortical layers (e.g. Layers 2/3) (Koenderink et al., 1994; Monique and Uylings, 1995). Postmortem analysis of brains from humans with ASD also report increased spine density on apical dendrites of pyramidal neurons compared to age matched, typically developing Controls, an effect most pronounced in Layer 2 (Hutsler and Zhang, 2010). Although impossible to compare rodent and human development at all levels, in terms of cortical maturation, P12-13 of a rodent approximates the period corresponding to full term birth in a human infant (Romijn et al., 1991). Human studies thus support our hypothesis that the PFC may be especially sensitive to environmental insults that occur after birth, such as sleep disruption.

4.3. ELSD decreases glutamate density, as well as nerve terminal and spine areas, within vGLUT1+ structures

Given our finding of increased spine density and spine immaturity within PL Layers 2/3 after ELSD, we next examined ultrastructural changes within glutamatergic synapses within Layers 2/3 of PL. We examined features of both pre- and post-synaptic contacts of terminals labeled with either one of two vesicular glutamate transporter types: vGLUT1 and vGLUT2. After ELSD, the density of glutamate immunogold particles, following exposure of the sectioned tissue to an antibody against glutamate, within vGLUT1-labeled terminals was significantly decreased. This effect was specific to vGLUT1, as it was not observed within vGLUT2-labeled terminals. Furthermore, ELSD decreased both presynaptic nerve terminal and post-synaptic spine area in vGLUT1-, but not vGLUT2-labeled terminals. This is consistent with our earlier findings of increased spine density and thinner dendritic spines in Layers 2/3 mPFC. Combined, these data suggest that ELSD results in a fundamental shift to an increase in smaller and more immature spines in ELSD animals (Mattison et al., 2014).

The specificity of our findings to only vGLUT1-labeled terminals may have several implications. First, it could suggest that corticocortical connections (vGLUT1), rather than thalamocortical connections (vGLUT2) may be preferentially affected by ELSD. This is consistent with our previous findings using Rapid Golgi staining showing a differential effect of ELSD in Layers 2/3, but not Layer 5. Second, vGLUT1 expression is crucially upregulated in the rodent cortex during the third postnatal week of development, corresponding to the start of ELSD, whereas cortical vGLUT2 is most abundant in the first two weeks of postnatal life (Fremeau et al., 2004a,b; Miyazaki
4.4. Potential mechanisms, limitations, and implications

Potential mechanisms explaining these findings could include one or both of the following possibilities: 1) increased wake during ELSD leads to increased glutamate in the brain (Bettendorff et al., 1996) with long term effects on developmentally-sensitive glutamatergic structures (Cruz-Martin et al., 2012), or 2) decreased REM or fragmented NREM sleep leads to decreased pruning of spines and impaired development of glutamatergic synapses (Cao et al., 2020).

There are several limitations to this study. One possible interpretation is that our effects could be due to the stress of ELSD. Although prior evidence suggests that changes to spine density on Layers 2/3 pyramidal neurons can occur due to stress or in response to corticosterone (Cook and Wellman, 2004; Seib and Wellman, 2003; Wellman, 2001), we have found no difference in anxiety-like behaviors or serum levels of corticosterone in voles that undergo ELSD compared to controls (Jones et al., 2019). Furthermore, most studies indicate spine loss in apical dendrites within Layers 2/3 of the mPFC (Brown et al., 2005; Cook and Wellman, 2004; Radley JJ et al., 2004; Radley Jason J et al., 2006) where our results are most consistent with spine growth or lack of pruning of spines after ELSD.

A second limitation is the focus on the third postnatal week of development. While this period has been implicated in many developmental studies across species, including for sleep ontogeny (Cao et al., 2020), vGLUT1/2 expression (Wojcik et al., 2004), and dendritic spine development within PFC (see (Kolb et al., 2012) for review), it is unclear whether this is a critical window or simply a sensitive period during development for effects of ELSD. Future work will examine ELSD at different developmental time points to answer this question. Our conclusions would be strengthened by direct measurement of sleep disruption in the animals used for this study. Although the current methods to record REM and NREM sleep in rodents involve surgical implantation of EEG and EMG electrodes (requiring anesthesia, handling, and separation from the dam and natal litter for neonates) the emergence and validation of less invasive techniques, including wireless electrodes and telemetry monitoring, may provide future studies an answer to these remaining questions.

Finally, a remaining question is how social behavior and cognitive flexibility, both hallmarks of ASD, integrate within the PFC not only in this vole model, but also in humans with ASD. It is possible individuals with impaired social function also have a similar degree of cognitive rigidity, and that a single circuit within the PFC underlies both complex behaviors (Kim et al., 2015; Milad and Quirk, 2002; Morgan et al., 1993; Murugan et al., 2017; Quirk et al., 2000; Selimbeyoglu et al., 2017; Sierra-Mercado et al., 2011; Yizhar et al., 2011). Future studies would need to examine both sociality and cognition within the same individual for a more comprehensive understanding of their interaction, and extend studies beyond glutamate to include commonly co-released neuropeptides such as oxytocin (see (Hrabovszky and Liposits, 2008)). For example, in prairie voles, activation of oxytocin receptors in the prelimbic region are necessary for social bonding (Young et al., 2001). Additionally, direct activation of glutamatergic neurons that express oxytocin receptors in PL alter social behaviors in mice (Tan et al., 2019). Interestingly, oxytocin receptor density peaks at P14 in the developing rodent cortex, with the densest distribution of receptors located in Layers 2/3 (Hammock and Levitt, 2013). Thus, future work should also examine patterns of oxytocin receptor development in PL and its relationship to glutamate after ELSD.

Our work has strong parallels with ASD. Neuropathological studies in patients with ASD have shown increased spine density within the cortex (Hutslar and Zhang, 2010). Additionally, sleep problems are highly prevalent in patients with ASD, include decreased time in REM and fragmented sleep (Buckley et al., 2010), an early life sleep pattern mirrored by our ELSD protocol. Although ASD is typically diagnosed around 3–5 years of age, behavioral and sleep deficits persist into adulthood. Emerging evidence suggests that sleep onset problems in the first year of life precede ASD diagnosis and are associated with altered neurodevelopment, consistent with our hypothesis that there is a causal role between early life sleep disruption and the later emergence of ASD (MacDuffie et al., 2020; Simola et al., 2014).

5. Conclusion

We have now shown, using the highly social prairie vole, that early life sleep disruption during the third postnatal week of development - a time period corresponding to the human first year of life - results in long term impairments in social behavior (Jones et al., 2019), reduced cognitive flexibility, and altered glutamatergic synapse structures within the medial prefrontal cortex (see Graphical Abstract). Our results support the hypothesis that early life experiences directly shape brain structure and function, with particularly outsized effects on late-maturing, experience-dependent regions such as the prefrontal cortex. Because human neurodevelopmental disorders including autism spectrum disorder also show poor sleep, impaired social behavior, cognitive inflexibility, as well as altered dendritic spine density and glutamate changes, our results have profound implications about the directionality and potential mechanisms underlying the relationship between early life sleep, neural plasticity, and behavioral development.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Peer Review Overview and Supplementary data

A Peer Review Overview and (sometimes) Supplementary data associated with this article can be found in the online version, at https://doi.org/10.1161/JCRNEU.2021.100020.

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