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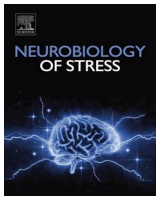

Functional and morphological adaptation of medial prefrontal corticotropin releasing factor receptor 1-expressing neurons in male mice following chronic ethanol exposure

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ABSTRACT

Chronic ethanol dependence and withdrawal activate corticotropin releasing factor (CRF)-containing GABAergic neurons in the medial prefrontal cortex (mPFC), which tightly regulate glutamatergic pyramidal neurons. Using male CRF1:GFP reporter mice, we recently reported that CRF1-expressing ($mPFC^{CRF1+}$) neurons predominantly comprise mPFC prelimbic layer 2/3 pyramidal neurons, undergo profound adaptations following chronic ethanol exposure, and regulate anxiety and conditioned rewarding effects of ethanol. To explore the effects of acute and chronic ethanol exposure on glutamate transmission, the impact of chronic alcohol on spine density and morphology, as well as persistent changes in dendritic-related gene expression, we employed whole-cell patch-clamp electrophysiology, diOlistic labeling for dendritic spine analysis, and dendritic gene expression analysis to further characterize $mPFC^{CRF1+}$ and $mPFC^{CRF1-}$ prelimbic layer 2/3 pyramidal neurons. We found increased glutamate release in $mPFC^{CRF1+}$ neurons with ethanol dependence, which recovered following withdrawal. In contrast, we did not observe significant changes in glutamate transmission in neighboring $mPFC^{CRF1-}$ neurons. Acute application of 44 mM ethanol significantly reduced glutamate release onto $mPFC^{CRF1+}$ neurons, which was observed across all treatment groups. However, this sensitivity to acute ethanol was only evident in $mPFC^{CRF1-}$ neurons during withdrawal. In line with alterations in glutamate transmission, we observed a decrease in total spine density in $mPFC^{CRF1+}$ neurons during dependence, which recovered following withdrawal, while again no changes were observed in $mPFC^{CRF-}$ neurons. Given the observed decreases in $mPFC^{CRF1+}$ stubby spines during withdrawal, we then identified persistent changes at the dendritic gene expression level in $mPFC^{CRF1+}$ neurons following withdrawal that may underlie these structural adaptations. Together, these findings highlight the varying responses of $mPFC^{CRF1+}$ and $mPFC^{CRF1-}$ cell-types to acute and chronic ethanol exposure, as well as withdrawal, revealing specific functional, morphological, and molecular adaptations that may underlie vulnerability to ethanol and the lasting effects of ethanol dependence.

1. Introduction

Alcohol use disorder (AUD) is a chronic, relapsing disorder, characterized by a compromised ability to regulate alcohol use despite adverse consequences. AUD is driven largely by complex adaptations in brain reward and stress systems (Koob, 2003, 2008; Becker and Lopez 2004) along with concomitant dysregulation of executive function by the medial prefrontal cortex (mPFC) (Pfarr et al., 2015; George et al., 2012; George and Koob 2011). Chronic alcohol exposure affects the structure and function of the prefrontal cortex, causing deficits in

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executive control, decision-making, and risk management (Abernathy et al. 2010; Pfarr et al., 2015; George and Koob 2011). Individuals with an AUD have reduced prefrontal cortex volumes (Jernigan et al., 1991; de la Monte 1988; Pfefferbaum et al., 1997; Harris et al., 2008; Kril et al., 1989), and mPFC hypo-functionality contributes to a loss of control over limiting alcohol consumption in individuals with AUD (Goldstein and Volkow 2011).

Preclinical studies further implicate the mPFC in anxiety-like behaviors and excessive alcohol drinking (Hare and Duman 2020; Klenowski 2018). The rodent mPFC is divided into the prelimbic (PL) and infralimbic (IL) subregions (Abernathy et al. 2010). Glutamatergic pyramidal neurons represent ~80% of the neurons and are tightly controlled by inhibitory GABA interneurons (Olah et al., 2009; Petersen and Crochet 2013). Our previous studies revealed that ethanol dependence induced by chronic intermittent ethanol (CIE) increases postsynaptic glutamate receptor function in mPFC PL, but not IL, layer 2/3 synapses and increases the proportion of mushroom spines (Varodayan et al., 2018). Moreover, withdrawal from CIE increases the excitability of layer 2/3 pyramidal neurons in mice (Pleil et al., 2015). Recently, we also established a role for mPFC AMPA receptors adaptations in the glutamatergic dysfunction associated with ethanol dependence (Siddiqi et al., 2023). However, understanding the precise role of discrete cell-types in the mPFC their contribution to AUD-related behavior is critical to gain mechanistic insight and develop targeted interventions for AUD.

The corticotropin-releasing factor (CRF) brain stress system plays a key role in excessive alcohol intake associated with ethanol dependence. Several studies have demonstrated the recruitment of CRF signaling in the central nucleus of the amygdala (CeA) underlying heighten anxiety-like behavior following withdrawal from chronic ethanol (Menzaghi et al., 1994; Koob 2008; Valdez et al., 2003; Funk et al., 2007; Chu et al., 2007). Using a transgenic mouse model expressing green fluorescent protein (GFP) under the CRF1 gene promoter (CRF1:GFP mice) (Justice et al., 2008), we identified unique molecular and functional properties distinguishing CeA CRF1 expressing (CeA^{CRF1+}) from unlabeled (CeA^{CRF1-}) neurons in ethanol dependence (Herman et al., 2013; Herman et al. 2016; Wolfe et al., 2019), but found no differences in CeA^{CRF1+} mature spines and basal glutamate transmission (Wolfe et al., 2019).

The CRF/CRF_1 system is also prominently expressed in the mPFC (Yan et al., 1998; Li et al., 2016; De Souza et al., 1985; Swanson et al., 1983; Uribe-Marino et al., 2016; Hu et al., 2011); CRF is co-expressed in predominantly vasoactive intestinal peptide-, although there is some co-expression in somatostatin- and parvalbumin-expressing GABA interneurons (Yan et al., 1998; Kubota et al., 2011; Gallopin et al., 2006; Taki et al. 2000; Chen et al., 2020). Notably, cell type-specific transcriptomics studies conducted on both adult rodents and humans have further validated these findings (Tasic et al., 2018). An additional source of CRF in the mPFC is from long-range inputs from the periventricular nucleus of the thalamus (Riad et al., 2022). In contrast, CRF_1 receptors are expressed in glutamate pyramidal neurons (Gallopin et al., 2006; Patel et al., 2022). Recruitment of CRF/CRF_1 signaling in the mPFC increases excitability of layer 2/3 mPFC neurons (Li et al., 2016) and excitatory postsynaptic currents (EPSCs) (Liu et al., 2015). Much less is known about the mPFC CRF system in AUD-related behaviors, although notably, ethanol withdrawal activates mPFC CRF containing GABAergic neurons (George et al., 2012). Recently, we found that mPFC CRF1 expressing ($mPFC^{CRF1+}$) neurons are densely expressed in mPFC layer 2/3 and undergo profound chronic ethanol-induced adaptations (Patel et al., 2022). Specifically, withdrawal from CIE (5–8 days into forced abstinence from CIE) dramatically decreases $mPFC^{CRF1+}$ excitability compared to naïve and dependent mice, pointing to the selective sensitivity of $mPFC^{CRF1+}$ to withdrawal (Patel et al., 2022). In contrast, non-labeled $mPFC^{CRF1-}$ neuronal excitability is increased in dependent and withdrawn mice. Using an ex vivo optogenetic approach we measured the functional connectivity between the basolateral amygdala (BLA) and $mPFC^{CRF1+}$ neurons and found that withdrawal-induced dysregulation in glutamate transmission in $mPFC^{CRF1+}$ is mediated in part by the BLA circuit (Patel et al., 2022). Additionally, through transcriptomic analyses, we found that $mPFC^{CRF1+}$ neurons exhibit heightened expression of the neuroimmune mediator colony-stimulating factor 1 (Csf1). Selective overexpression of Csf1 in $mPFC^{CRF1+}$ neurons could reduce postsynaptic glutamate transmission, offering a mechanistic understanding of the synaptic changes triggered by withdrawal in $mPFC^{CRF1+}$ neurons (Patel et al., 2022).

Dendritic geometry is a key determinant of neurotransmission (Mainen and Sejnowski 1996; Krichmar et al., 2002; Vetter et al. 2001), and potentiation of excitatory transmission is associated with an increase in spine size (Yuste and Majewska 2001; Alvarez et al. 2007). Thus, in the current study, we assessed potential cell-type specific ($mPFC^{CRF1+}$ vs $mPFC^{CRF1-}$) morphological/structural alterations that may drive the synaptic functional changes induced by ethanol dependence and withdrawal and identified a unique transcriptomic signature, including gene-networks and ontologies enriched in the $mPFC^{CRF1+}$ population in the mPFC.

2. Materials and Methods

2.1. Animals

All procedures involving the use of experimental animals in this study were approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adult, male (minimum age of 10 weeks) CRF1:GFP mice ($n = 62$) were bred in-house. Mice were group-housed in a temperature and humidity-controlled vivarium on a 12-h reversed light/dark cycle (lights turn off at 8am) with food and water available *ad libitum*.

2.2. Chronic intermittent ethanol exposure

Five different cohorts of male CRF1:GFP mice were subjected to the chronic intermittent ethanol inhalation (CIE) paradigm to induce ethanol dependence in male mice by exposing them to 5 to 6 consecutive weeks of ethanol vapor as previously reported (Herman et al. 2016; Patel et al. 2019, 2021, 2022). Briefly, mice in the ethanol dependent group were intraperitoneally injected with 1.75 g/kg ethanol +68.1 mg/kg pyrazole (alcohol dehydrogenase inhibitor) and placed in vapor chambers for 4 days (16 h vapor on, 8 h off). Every week of ethanol exposure, on the third or fourth day of vapor exposure tail blood was collected to determine blood ethanol levels. The average blood ethanol level achieved was 184.80 ± 5 mg/dl ($n = 42$) during the weeks of ethanol vapor exposure. Naïve mice ($n = 20$) were injected with 68.1 mg/kg pyrazole in saline and were exposed to air in identical cages. Experiments were conducted from naïve and dependent (also defined as CIE) mice directly from the vapor chambers, while the withdrawn (also defined as WD) mice were 5–8 days into forced abstinence.

2.3. Whole-cell patch clamp electrophysiology in ex vivo brain slices

2.3.1. Slice preparation

Mice were anesthetized with 3–5% isoflurane, rapidly decapitated, and brains were rapidly removed and placed in ice-cold oxygenated (95% O2 and 5% CO2) high-sucrose cutting solution (Varodayan et al., 2023) containing (in mM): 206 sucrose, 2.5 KCl, 2.5 CaCl2, 7 MgCl2, 1.2 NaH2PO4, 26 NaHCO3, 5 glucose, 5 HEPES; pH 7.3. Coronal slices (300-μM) containing the mPFC were cut using a 1200S vibratome (Leica Microsystems, Buffalo Grove, IL) and incubated in artificial cerebrospinal fluid (aCSF) containing (in mM): 130 NaCl, 3.5 KCl, 1.25 NaH2PO4, 1.5 MgSO4, 2 CaCl2, 24 NaHCO3, 10 glucose; pH 7.3 at 32 °C for 30 min and then at room temperature for at least 30 min before use.

2.3.2. Whole-cell electrophysiological recordings

We visualized neurons in the mPFC PL subdivision with infrared differential interference contrast (IR-DIC) optics, and we identified GFP labeled neurons ($mPFC^{CRF1+}$) using Prior LED optics (Prior Scientific, Rockland, MA). Whole-cell voltage-clamp and current-clamp recordings were obtained using Multiclamp 700B amplifier, Digidata 1440A, and pClamp 10 software (Molecular Devices, Sunnyvale, CA). Glass pipettes were pulled using PC-10 puller (Narishige International USA, Amityville, NY) to a resistance of 3–6 MΩ and filled with an internal solution containing (in mM): 145 K-gluconate, 0.5 EGTA, 2 MgCl2, 10 HEPES, 2 Mg-ATP, 0.2 Na-GTP; 290–300 mOsms; pH 7.2–7.3. Glutamatergic spontaneous excitatory postsynaptic currents (sEPSCs) were recorded using a holding potential of −70mV in the presence of 30 μM bicuculline (BIC; Sigma-Aldrich, St. Louis, MO) and 1 μM [1-(S)-3,4-dichlorophenyl) ethyl]amino-2-(S)-hydroxypropyl-p- benzylphosphonic acid (CGP 52432) (Tocris, Bio-Techne Corporation, Minneapolis, MN) for patch clamp experiments. Series resistance was not compensated, and cells with a series resistance >20 MΩ or with a >20% change during the recording were excluded.

We used Mini Analysis (Synaptosoft Inc., Fort Lee, NJ) to analyze sEPSC properties (frequency, amplitude, rise time and decay time) and visually confirmed. Events <5 pA were excluded from the analysis, and the average properties from a minimum of 60 events during a 3 min interval were calculated. To control for cell-to-cell variation in baseline electrophysiology properties, the effects of acute ethanol application at the concentration of 44 mM (Roberto et al. 2003, 2004; Kallupi et al., 2014; Herman et al., 2016b) were normalized to their own neuron's baseline prior to group analyses. We used ethanol at 44 mM. a maximal concentration, based on our concentration response studies (Roberto et al. 2003, 2004; Varodayan et al., 2023). All final values were analyzed for significance using one-sample *t*-tests and compared using paired *t*-tests or one-way ANOVAs with a *post hoc* Tukey's multiple comparisons test where appropriate, using Prism 10.2. (GraphPad, San Diego, CA). Generally, decreased sEPSC frequencies are associated with lower glutamatergic release, while changes in amplitude and kinetics are linked to altered postsynaptic receptor function (Otis et al., 1994). Bicuculline (Sigma-Aldrich), CGP 52432 (Tocris) and ethanol (Remet, La Mirada, CA) were applied by ACSF bath perfusion.

2.4. Dendritic spine analysis

2.4.1. Tissue preparation

Male mice (n = 3–4 per group) were anesthetized using isoflurane and perfused with cold 4% paraformaldehyde (PFA) made in phosphate buffered saline (PBS). Brains were extracted and immersion fixed in 4% PFA at 4 °C for 2 h before being coronally sectioned into 100-μm slices on a vibrating microtome (Leica VT1000S, Leica Microsystems).

2.4.2. Diolistic labeling

Sections were biolistically labeled with DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate)-coated 1.1-μm tungsten particles delivered with a Bio-Rad gene gun and incubated in PBS overnight at 4 °C before immunostaining (Wolfe et al., 2019). Slices were permeabilized in 0.01% Triton X-100 for 15 min at room temperature (RT) with gentle agitation and blocked for 30 min at RT with gentle agitation in blocking solution (10% normal goat serum (NGS) in 0.01% Triton X-100) (Wolfe et al., 2019). Primary antibody incubation was performed overnight at 4 °C in PBS (chicken anti-GFP; Abcam, ab13970, RRID:AB_300798; 1:2000). The slices were triple washed with PBS for 10 min followed by secondary antibody incubation for 1 h at RT in blocking solution (Alexa Fluor 488 goat anti-chicken; Thermo Fisher Scientific, A-11039, RRID:AB_142924; 1:1000). The slices were triple washed in PBS and mounted on slides with Prolong Diamond Antifade Mountant (Thermo Fisher Scientific, Waltham, MA; P36965).

2.4.3. Imaging and analysis

Slides were imaged on a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss MicroImaging; 63 × oil immersion NA 1.4, 1024 × 1024, 1-μm step z-stacks). All microscope settings were kept the same within experiments during image acquisition. Imaris v 9.9.0 (Oxford Instruments) was used to quantify filopodia, thin, stubby, and mushroom-shaped dendritic spines in both $mPFC^{CRF1+}$ (n = 13 neurons, 37 dendritic segments) and $mPFC^{CRF1-}$ neurons (n = 24 neurons, 41 dendritic segments) (Wolfe et al., 2019). Spines were classified using the Imaris MatLab Xtension "Classify Spines" using the default parameters. Spine classification was independently validated manually. The experimenter was blind to cell-type ($mPFC^{CRF1+}$ versus $mPFC^{CRF1-}$) and animal condition when performing spine quantification. Data obtained in each mouse were averaged for each cell-type, such that the number of mice was used as the n in statistical analyses.

2.5. Analysis of differentially expressed dendritic genes in $mPFC^{CRF1+}$ neurons

Previously, RNA sequencing was performed on FACS sorted $mPFC^{CRF1+}$ neurons from male ethanol naïve and CIE withdrawal mice (Patel et al., 2022). The differentially expressed genes (DEGs) from this analysis were used to identify potential molecular contributors to the observed alterations in spine density and morphology in the current study. We utilized publicly available RNA sequencing datasets that identified genes enriched in dendrites compared to neuronal cell bodies (Middleton et al. 2019; Kratz et al., 2014; Cajigas et al., 2012; Ainsley et al., 2014; Hobson et al., 2022; Lein et al., 2007; Poon et al., 2006; Taliaferro et al., 2016; Tushev et al., 2018). A list of overlapping genes was identified between the DEGs of mPFCCRF1+ neurons and dendritic genes identified in at least two of these publications. Metascape (Zhou et al., 2019) analysis and manually curated functional groups of genes were used to investigate the function of the identified set of mPFCCRF1+ dendritic genes. To understand the functional involvement of the dysregulated dendritic genes we used 2 different approaches, Metascape (Zhou et al., 2019) to gain unbiased understanding of the pathways those genes are involved in (Fig. 4D), and manual curation of the genes function and annotated functions relevant to synapses structure (extracellular matrix, cytoskeleton) and function (neurotransmitter release mechanism and calcium signaling).

2.6. Statistical analyses

Electrophysiological measures were analyzed by one-way ANOVA and one sample *t*-test. Spine densities and spine type proportions in $mPFC^{CRF1+}$ and $mPFC^{CRF1-}$ neurons were compared using one-way ANOVA, with spine shape as the within-subject variable and treatment as the between-subject variable and using paired t tests. For all statistical analyses, including the normality tests, we used GraphPad Prism 10 software (San Diego, CA). The results of the normality tests are presented in the supplementary materials. Data are presented as mean ± standard error of the mean (SEM), with the number of cells and mice used for each experiment reported in the figure legend. In all cases, $p < 0.05$ was the criterion for statistical significance.

3. Results

3.1. Ethanol dependence enhances spontaneous glutamatergic transmission in $mPFC^{CRF1+}$ prelimbic layer 2/3 neurons

To investigate potential neuroadaptations in glutamatergic transmission within the $mPFC^{CRF1+}$ prelimbic (PL) layer 2/3 neurons following ethanol dependence and withdrawal (see Schematic, Fig. 1A), we used whole-cell patch clamp electrophysiology to record spontaneous excitatory postsynaptic currents (sEPSCs), which contain both action potential-dependent and action potential-independent

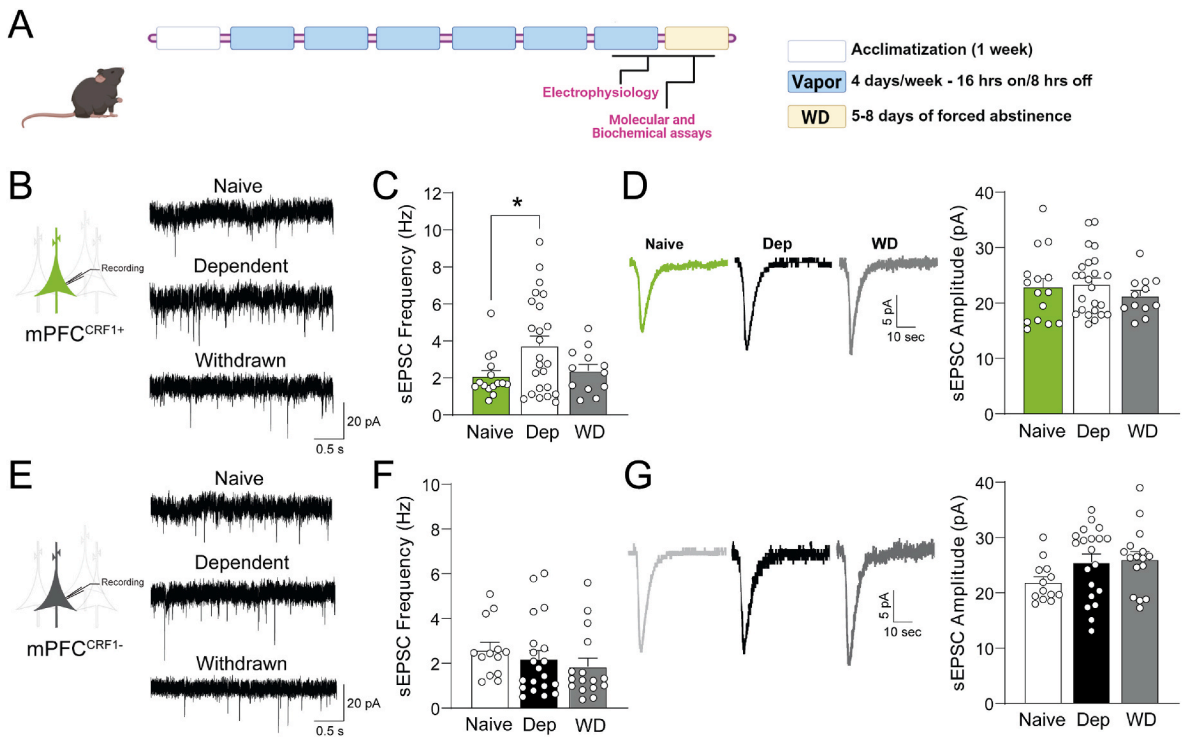

Fig. 1. mPFC^{CRF1+} neurons show elevated basal spontaneous glutamatergic transmission following ethanol dependence. A. Schematic of chronic intermittent ethanol exposure and withdrawal (made using Biorender). **B.** Representative spontaneous excitatory postsynaptic currents (sEPSC) traces obtained from mPFC^{CRF1+} PL layer 2/3 neurons of naïve, dependent (Dep), and withdrawn (WD) male mice. **C.** Average sEPSC frequency showing increased sEPSC frequency in mPFC^{CRF1+} of dependent mice. **D.** Average sEPSC amplitude from mPFC^{CRF1+} showing no difference across groups. Right panel: scaled sEPSC traces. Left panel: mean sEPSC amplitude histograms. **E.** Representative sEPSC traces obtained from mPFC^{CRF1-} PL layer 2/3 neurons of naïve, dependent, and withdrawn male mice. **F.** Average sEPSC frequency showing no difference across groups. **G.** Average sEPSC amplitude from mPFC^{CRF1-} showing no difference across groups. Left panel: scaled sEPSC traces. Right panel: mean sEPSC amplitude histograms. n = 12–24 cells from N = 8–11 mice per group. *$p < 0.05$ by one-way ANOVA and Tukey's post hoc test.

neurotransmission. We found that chronic ethanol exposure alters basal sEPSC frequencies (one-way ANOVA, $F_{(2, 48)} = 3.72$, *$p = 0.03$; Tukey's multiple comparison test, *$p = 0.04$) with mPFC^{CRF1+} neurons of ethanol dependent mice displaying significantly higher sEPSC frequencies (3.73 ± 0.5 Hz; n = 24; Fig. 1A–C), suggesting they receive more glutamatergic input (glutamate release), compared to naïve mice (2.10 ± 0.3 Hz; $n = 15$; Fig. 1B and C). Notably, in mPFC^{CRF1+} neurons of withdrawn mice (5–8 days into forced abstinence) the mean sEPSC frequency (2.4 ± 0.4 Hz; n = 12; Fig. 1B and C) was similar to naïve mice. We did not observe significant changes in the amplitude, rise time, and decay time (Fig. D, Table 1) in mPFC^{CRF1+} neurons of dependent and withdrawn mice. We also recorded from neighboring mPFC^{CRF1-} neurons and found that ethanol dependence and withdrawal did not alter sEPSC properties (Fig. 1E–G, Table 1). Overall, two-way ANOVA revealed no significant effect for a cell type by ethanol treatment interaction for sEPSC frequency ($F(2, 94) = 2.8$, $p = 0.06$) or amplitude ($2, 94) = 1.7$, $p = 0.17$). Generally, increased sEPSC frequencies reflect higher presynaptic glutamate release and synaptic connections, whereas increased amplitudes and kinetics indicate enhanced glutamate receptor sensitivity (via changed receptor number and/or subunit composition) and synapse number (De Koninck and Mody, 1994; Otis et al., 1994).

3.2. Acute ethanol application decreases glutamate release onto mPFC^{CRF1+} prelimbic layer 2/3 neurons in naïve, ethanol dependent, and withdrawn mice

In a subset of recorded neurons, we investigated the effects of acute, 44 mM (Roberto et al., 2004; Kallupi et al., 2014; Herman et al., 2016b) ethanol application (12–15 min) on mPFC^{CRF1+} and mPFC^{CRF1-} PL layer 2/3 populations in the naïve, dependent, and withdrawn mice. We found ethanol significantly (one sample t-test, $p < 0.01$) decreased sEPSC frequency in mPFC^{CRF1+} neurons of naïve mice (75.2 ± 5.3% of baseline, n = 21), suggesting a decrease in presynaptic glutamate release. Similar ethanol-induced decreases in glutamate release were observed in mPFC^{CRF1+} of dependent (81.34 ± 4.1% of baseline, $n = 12$) and withdrawn mice (64.9 ± 8.3% of baseline, n = 8; Fig. 2A–C). No changes in sEPSC amplitude, rise time, and decay time were observed with acute ethanol application. (Fig. 2C–Table 2). When comparing the

Table 1
Baseline spontaneous excitatory post-synaptic potential characteristics for mPFC^{CRF1+} and mPFC^{CRF1-} neurons from naïve, dependent, and withdrawn mice. Statistical significance *$p < 0.05$ from one-way ANOVA Tukey's post hoc comparison to naïve.

		n	Frequency (Hz)	Amplitude (pA)	Rise time (ms)	Decay time (ms)
mPFC^{CRF1+}	Naïve	15	2.10 ± 0.30	22.75 ± 1.65	0.89 ± 0.08	0.92 ± 0.12
	Dependent	24	3.73 ± 0.53*	23.39 ± 1.12	0.87 ± 0.04	0.892 ± 0.08
	Withdrawn	12	2.39 ± 0.35	21.27 ± 1.02	0.88 ± 0.10	1.012 ± 0.16
mPFC^{CRF1-}	Naïve	13	2.60 ± 0.35	21.90 ± 1.00	1.06 ± 0.11	1.09 ± 0.18
	Dependent	20	2.19 ± 0.38	25.58 ± 1.46	1.03 ± 0.05	1.15 ± 0.12
	Withdrawn	16	1.85 ± 0.38	26.04 ± 1.45	1.04 ± 0.07	1.11 ± 0.14

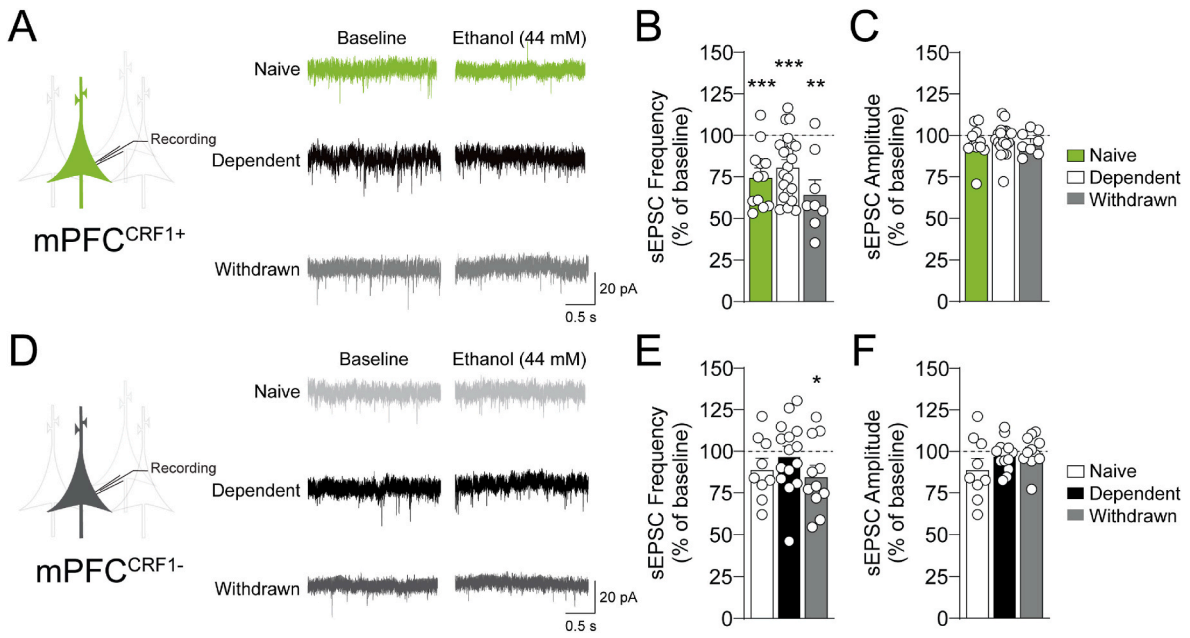

Fig. 2. Acute ethanol (44 mM) decreases glutamate release onto mPFC^{CRF1+} neurons in naïve, dependent, and withdrawn mice, which is only observed in mPFC^{CRF1-} neurons following withdrawal. A. Representative spontaneous excitatory postsynaptic currents (sEPSC) traces before and during ethanol application from mPFC^{CRF1+} neurons of naïve, dependent, and withdrawn male mice. **B.** Acute ethanol significantly decreases sEPSC frequencies in naïve, dependent, and withdrawn mice in mPFC^{CRF1+} neurons. **C.** Acute ethanol did not alter sEPSC amplitude in mPFC^{CRF1+} neurons. **D.** Representative sEPSC traces before and during ethanol application from mPFC^{CRF1-} neurons of naïve, dependent, and withdrawn mice. **E.** In mPFC^{CRF1-} neurons, acute ethanol significantly decreased the sEPSC frequency selectively in withdrawn mice. **F.** Acute ethanol did not alter sEPSC amplitude in mPFC^{CRF1-} neurons. n = 8–20 cells from N = 8–11 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001 by one-sample t-test.

Table 2
Spontaneous excitatory post-synaptic potential characteristics following 44 mM ethanol application for mPFC^{CRF1+} and mPFC^{CRF1-} neurons from naïve, dependent, and withdrawn mice. Statistical significance *p < 0.05, **p < 0.01, and ***p < 0.001 from one-sample t-test.

		n	Frequency (% of baseline)	Amplitude (% of baseline)	Rise time (% of baseline)	Decay time (% of baseline)
mPFC^{CRF1+}	Naïve	12	75.16 ± 5.32***	95.37 ± 2.86	91.51 ± 4.29	90.53 ± 7.06
	Dependent	21	81.34 ± 4.15***	97.48 ± 1.89	99.34 ± 2.59	100.7 ± 3.13
	Withdrawn	8	64.93 ± 8.35**	95.71 ± 2.45	93.78 ± 5.26	99.79 ± 8.02
mPFC^{CRF1-}	Naïve	9	89.46 ± 6.30	98.51 ± 2.18	98.07 ± 4.33	96.94 ± 6.81
	Dependent	15	97.53 ± 5.49	97.90 ± 2.23	99.96 ± 3.40	100.9 ± 4.23
	Withdrawn	11	85.22 ± 6.51*	99.64 ± 2.91	97.48 ± 5.54	95.11 ± 7.94

effects of ethanol across all three groups (naive, dependent, and withdrawn) via one-way ANOVA, we found no significant differences in the ethanol-induced decrease in sEPSC frequency across groups (p = 0.15).

Notably, acute ethanol did not alter sEPSC properties in mPFC^{CRF1-} PL neurons of naïve and dependent mice (n = 9). Acute ethanol decreased the frequency (85.2 ± 6.5% of baseline, n = 11; p < 0.05), but not amplitude or kinetics of sEPSCs, in mPFC^{CRF1-} neurons of withdrawn mice (Fig. 2D–F, Table 2). One-way ANOVA did not reveal any significant acute ethanol effects across groups for sEPSC frequency (p = 0.32). Two-way ANOVA showed a significant main effect of cell type (F(1,70) = 11.98, ***p = 0.0009) and ethanol treatment (F(2, 70) = 3.13, *p = 0.05) for sEPSC frequency, and no significant effects for sEPSC amplitude.

These data show that mPFC^{CRF1+} neurons are more sensitive to the acute effects of ethanol which decreases glutamate release onto this population, and that withdrawal induced neuroadaptations in mPFC^{CRF1-} lead to increased sensitivity to acute ethanol.

3.3. Chronic ethanol induces distinct morphological changes in mPFC^{CRF1+}, compared to mPFC^{CRF1-}, prelimbic layer 2/3 neurons in naïve, ethanol dependent, and withdrawn mice

To determine if chronic ethanol exposure and withdrawal lead to structural changes in mPFC^{CRF1+} and mPFC^{CRF1-} neurons, we collected mPFC tissue from naïve, dependent, and withdrawn mice, performed diolistic labeling, and dendritic spine analysis. We analyzed the morphology of dendritic protrusions, which were classified as mushroom (most mature), stubby, long/thin and filopodia (least mature; Fig. 3). In dependent mice (0.7 ± 0.26; n = 17), we observed a significant decrease in total spine density in mPFC^{CRF1+} neurons compared to naïve mice (1.2 ± 0.33; n = 12; one-way ANOVA, $F_{(2,39)}$ = 13.47, p < 0.0001; Fig. 3A and B). This reduction in total spine density was driven by a significant decrease in filopodia (one-way ANOVA with Tukey's multiple comparison's test, $F_{(2,39)}$ = 5.33, p < 0.01; mPFC^{CRF1+} naïve: 0.34 ± 0.05, n = 12 vs dependent: 0.17 ± 0.02, n = 17, p < 0.01; Fig. 3A–C), long/thin spines (one-way ANOVA with Tukey's multiple comparison's test, $F_{(2,39)}$ = 6.94, p < 0.01; naive: 0.44 ± 0.06, n = 12 vs dependent: 0.24 ± 0.02, n = 17, p < 0.01; Fig. 3D), and stubby spines (one-way ANOVA with Tukey's multiple comparison's test, $F_{(2,39)}$ = 5.6, p < 0.01; Fig. 3E); naïve: 0.26 ± 0.03, n = 12 vs dependent: 0.16 ± 0.02, n = 17, p < 0.01).

Interestingly, after withdrawal, the total spine density in mPFC^{CRF1+} neurons was comparable with naïve mice (Fig. 3A–F). Withdrawn mice showed no difference compared to naïve in spine types, except a significant decrease in stubby spines (one-way ANOVA with Tukey's multiple comparison's test, $F_{(2,39)}$ = 5.6, p < 0.05; naive: 0.22 ± 0.07, n = 17

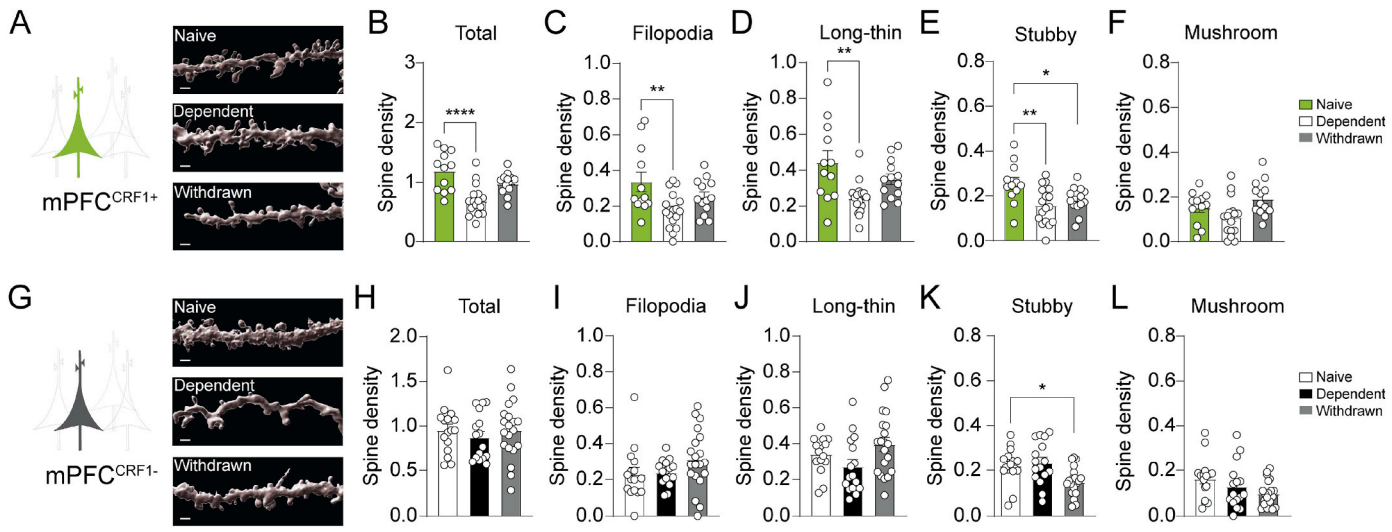

Fig. 3. mPFC^{CRF1+} and mPFC^{CRF1-} neurons show altered spine density in ethanol dependent and withdrawn mice. A. Representative images depicting spines from mPFC^{CRF1+} neurons of naïve, dependent, and withdrawn male mice. **B.** Average spine density in mPFC^{CRF1+} neurons of naïve, dependent, and withdrawn mice. **C–F.** Average total spine density for (C) filopodia, (D) long-thin, (E) stubby, and (F) mushroom spines in mPFC^{CRF1+} neurons of naïve, dependent, and withdrawn mice. **G.** Representative images depicting spines from mPFC^{CRF1-} neurons of naïve, dependent, and withdrawn male mice. **H.** Average total spine density per micron in mPFC^{CRF1-} neurons of naïve, dependent, and withdrawn mice. **I-L.** Average spine density for (I) filopodia, (J) long-thin, (K) stubby, and (L) mushroom spines in mPFC^{CRF1-} neurons of naïve, dependent, and withdrawn mice. $n = 12–20$ neurons from $N = 3–6$ mice per group. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ by one-way ANOVA and Tukey's post hoc test compared to naive. Scale bar = 2 μm.

vs withdrawn: 0.14 ± 0.06, $n = 13$), $p < 0.05$; Fig. 3E) was observed in withdrawn compared to naive mice.

In contrast, mPFC^{CRF1-} neurons showed no significant changes in total spine density with dependence or withdrawal (Fig. 3G and H). However, mPFC^{CRF1-} neurons displayed a significant decrease in stubby spines in withdrawal (one-way ANOVA, $F_{(2,49)} = 6.33$, $p < 0.01$; naive: 022 ± 0.07, $n = 16$ vs withdrawn: 0.15 ± 0.01, $n = 20$, $p < 0.01$; Fig. 3K). Using two-way ANOVA, there was a significant interaction effect F(2, 88) = 4.75, *p = 0.01 and main effect of ethanol treatment F (2, 88) = 9.42, p = 0.0002 for total spine density, demonstrating a significant difference between naive and dependent mPFC^{CRF1+} as well as dependent and withdrawn mPFC^{CRF1+} groups. These observations suggest that mPFC^{CRF1+} neurons undergo unique changes in structural plasticity to chronic ethanol dependence compared to mPFC^{CRF1-} neurons.

3.4. Dysregulation of dendritic genes in mPFC^{CRF1+} neurons during withdrawal

Our data shows persistent structural and electrophysiological changes in mPFCCRF1+ neurons following ethanol dependence. To understand the molecular changes mediating or associated with the observed decreases in stubby spines during withdrawal, we utilized our published dataset (Patel et al., 2022) in which we investigated gene changes of CRF1+ neurons during withdrawal, to investigated potential alterations in dendritic genes during withdrawal. To do this, we identified an overlapping set of 1674 dendritic genes that were reported in at least two dendritic gene profiling publications (see Materials and Methods; Fig. 4). We identified 510 downregulated and 2037 upregulated genes in ethanol withdrawal mPFC^{CRF1+} neurons (nominal p-value <0.05). mPFC^{CRF1+} differentially expressed genes (DEGs) showed significant overlap with the dendritic genes (Representation factor 1.4, p < 5.934e-05, hypergeometric test). 48 genes overlapped between the dendritic genes and mPFC^{CRF1+} upregulated genes, and 67 genes overlapped between the dendritic genes and mPFC^{CRF1+} downregulated genes (Fig. 4B). Manual curation and Metascape analysis of ethanol withdrawal dendritic genes show their association with canonical markers of neurotransmitter secretion, cytoskeleton and extracellular matrix, calcium signaling, protein methylation, neurofilament cytoskeleton organization, and neuron projection development associated processes (Fig. 4C and D).

To examine the functional relevance of the most alcohol sensitive dendritic gene, we investigated dysregulated dendritic genes with the highest fold changes (Fig. 4A). *Bgn*, a gene encoding biglycan, a structural component of the extracellular matrix and an inflammasome activator (Nastase et al. 2012), was upregulated in mPFC^{CRF1+} neurons during withdrawal. *Cachd1*, a gene encoding cache domain containing 1, is involved in calcium ion transmembrane transport and part of the voltage-gated calcium channel (Ferron et al. 2021). *Hsd17b14* encodes for a subtype of 17β-Hydroxysteroid dehydrogenases, a critical component in the metabolism of neurosteroids (He et al. 2019). *HSD17B14* expression regulates microglia-mediated inflammation (Kaoru Saijo et al., 2011). Top dendritic downregulated genes included *Rnaseh2a* which encodes ribonuclease H2 subunit A, a major source of ribonuclease H activity in mammalian cells. Together, the analysis of dendritically associated genes in mPFC^{CRF1+} neurons during ethanol withdrawal provides insights into mechanisms underlying vulnerability to ethanol and on the long-term effects of ethanol dependence, revealing associations with neurotransmitter secretion, cytoskeleton dynamics, calcium signaling, and neuroinflammation.

4. Discussion

Our previous studies have identified mPFC CRF1^{+} prelimbic layer 2/3 neurons as a distinct glutamatergic population regulating anxiety and conditioned ethanol reinforcement. Additionally, these neurons exhibit a unique electrophysiological signature compared to neighboring mPFC CRF1^{-} neurons in mice (Patel et al., 2022). Specifically, mPFC CRF1^{+} show reduced excitability, less voltage sag, depolarized resting membrane potential, as well as reduced sEPSC frequency and amplitude. Notably, while we did not measure excitability, the current study recapitulates our previous finding of reduced sEPSC frequency in mPFC CRF1^{+} compared to mPFC CRF1^{-}. In an additional study, we found that one week of withdrawal from chronic ethanol exposure induces morphological (increased spine maturity) and functional (increased postsynaptic glutamate receptor-mediated currents) changes in

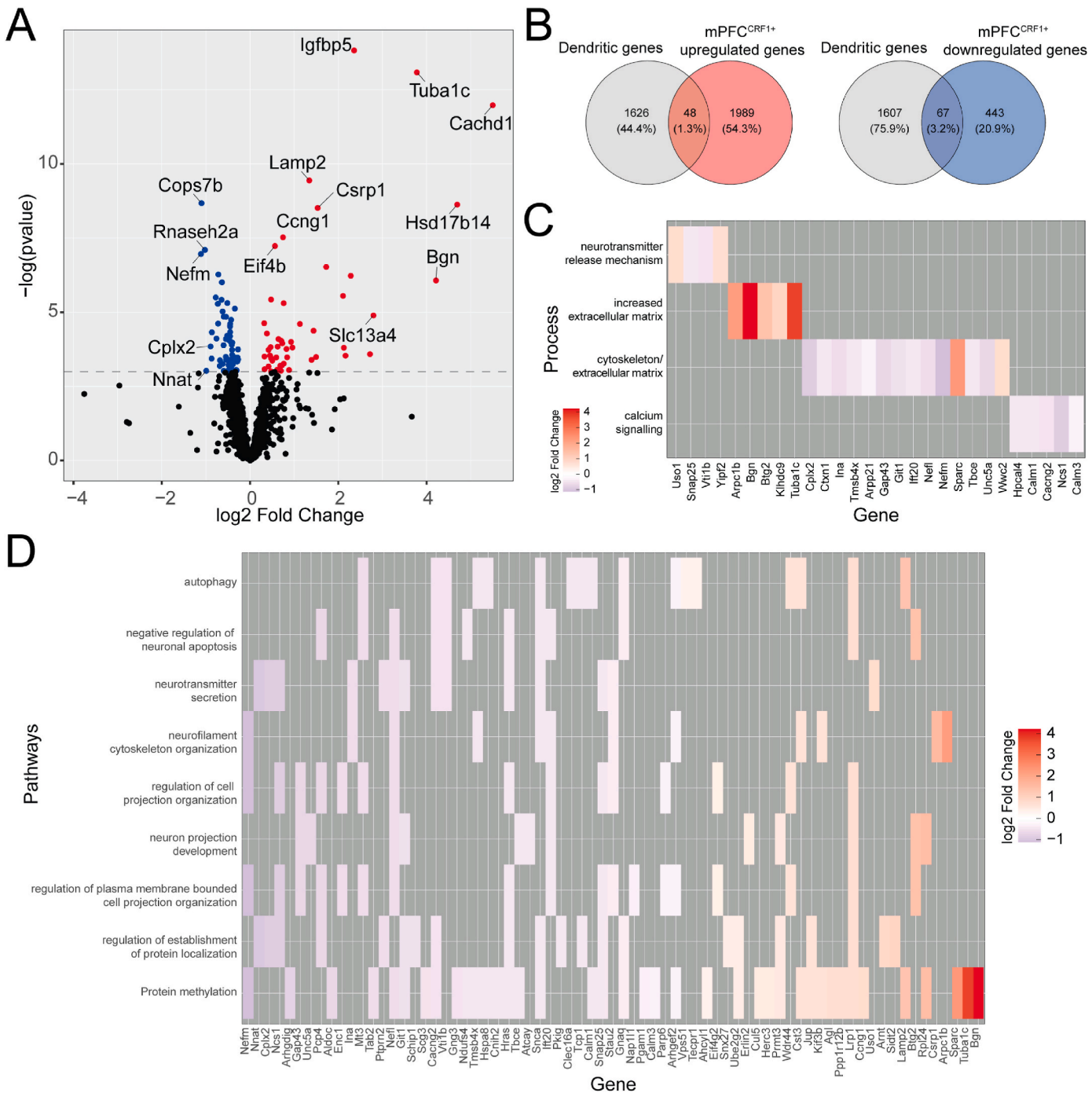

Fig. 4. Differential expression of predicted dendritic genes in mPFC^{CRF1+} neurons from ethanol withdrawn mice. A. Differential gene expression of predicted dendritic genes, showing upregulated (red) and downregulated (blue) genes in mPFC^{CRF1+} neurons from ethanol withdrawn compared to naïve mice. **B.** Overlap between dendritic genes, and upregulated (left Venn diagram) and downregulated genes (right Venn diagram) in mPFC^{CRF1+} neurons from ethanol withdrawn compared to naïve mice. **C.** Manual curation of canonical gene function for differentially expressed predicted dendritic genes, color indicates the differential expression fold change in ethanol withdrawn compared to naïve mice. **D.** Top Reactome and GO Biological Processes identified by Metascape analysis for differentially expressed predicted dendritic genes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

unlabeled prelimbic layer 2/3 pyramidal neurons of the mPFC (Varodayan et al., 2018). Building on these findings, the goal of our study was to investigate the specific functional and morphological adaptations occurring during and after chronic ethanol dependence in mPFC^{CRF1+} and mPFC^{CRF1-} prelimbic neurons of male CRF1:GFP mice. Functionally, we found that mPFC^{CRF1+} neurons are sensitive to ethanol dependence, showing increased excitatory glutamate release. However, glutamate transmission in mPFC^{CRF1-} neurons was unaltered by dependence and withdrawal. Notably, mPFC^{CRF1+} neurons show decreased glutamate release with acute ethanol, not observed in mPFC^{CRF1-}, which persisted in this population during dependence and withdrawal. In line with these findings, mPFC^{CRF1+} neurons displayed decreased spine density and type alterations during dependence, which persisted for stubby spines into withdrawal. In contrast, spine density in mPFC^{CRF1-} neurons was unaltered by dependence and withdrawal. To better understand the enduring changes in mPFC^{CRF1+} neurons

following ethanol dependence, we examined dendritic gene expression in mPFC^{CRF1+} neurons from withdrawn compared to naïve mice and found associations with neurotransmitter secretion, cytoskeleton dynamics, calcium signaling, and neuroinflammation. These data highlight the sustained dendritic effects of ethanol dependence on mPFC^{CRF1+} neurons during withdrawal that may contribute to their sensitivity to ethanol. In summary, our study highlights the differential responses of mPFC^{CRF1+} and mPFC^{CRF1-} prelimbic neurons to acute and chronic ethanol exposure as well as withdrawal, highlighting the specific functional and morphological adaptations that occur in these neuronal populations.

Growing literature supports that chronic ethanol consumption differentially impacts glutamatergic transmission in the cortical areas with consequences on craving, cognitive deficits, and AUD severity in patients. In our functional analysis, we observed that mPFC^{CRF1+} neurons of dependent mice receive significantly higher glutamate release compared to naïve mice. Interestingly, this effect reversed following approximately a week of withdrawal, which is consistent with our previous results (Patel et al., 2022). Notably, ethanol dependence and withdrawal did not affect glutamatergic transmission in neighboring mPFC^{CRF1-} neurons, indicating a selective sensitivity of mPFC^{CRF1+} neurons to ethanol dependence, although we previously found increased glutamate release on mPFC^{CRF1-} neurons during withdrawal (Patel et al., 2022). In addition, in the current study, we did not observe changes in amplitude or postsynaptic properties in either this mPFC^{CRF1+} or mPFC^{CRF1-} neuronal populations in dependent and withdrawn mice. This contrasts with our previous studies that found decreased postsynaptic glutamate transmission on mPFC^{CRF1+} neurons selectively in withdrawal as well as increased postsynaptic glutamate transmission on the mPFC^{CRF1-} neurons with dependence and withdrawal (Patel et al., 2022). Understanding the effects of ethanol consumption is crucial but can be quite complex and vary depending on length of alcohol drinking, pattern of alcohol exposure, amount of alcohol consumed, and time of alcohol abstinence. In another study, we found that mice in 1-week of withdrawal from 7 weeks of CIE-exposure displayed a similar sEPSC frequency to that of control mice, but had significantly higher sEPSC amplitude and kinetics (Varodayan et al., 2018; Siddiqi et al., 2023; De Koninck and Mody 1994; Otis et al. 1994) These discrepancies may be due to differences in the length of the chronic ethanol vapor exposure (5 versus 7 weeks) (Patel et al., 2022; Varodayan et al., 2018). Similar conflicting reports exist in the literature. For instance, while some studies indicate increased glutamate signaling in individuals with AUD (Bauer et al., 2013), others report a decrease (Ende et al., 2013). In addition, preclinical studies in mice and rats provide conflicting data on the impact of chronic ethanol exposure on the glutamatergic functions of the mPFC (Pleil et al., 2015; Kroener et al., 2012; Joffe et al., 2021; Galaj et al., 2020; Gass et al., 2014b) as well as morphological changes, such as alterations in spine density (Kim et al., 2015; Varodayan et al., 2018; Szumlinski et al., 2023; Holmes et al., 2012). However, focusing on the CIE model of alcohol dependence, a defining feature observed across multiple studies, including the current study, is an increase in glutamatergic transmission in the mPFC (Pleil et al., 2015; Patel et al., 2022; Varodayan et al., 2018; Siddiqi et al., 2023). This may represent a critical neuroadaptation, not consistently observed with other forms of alcohol exposure, that may contribute to alcohol dependence-related behaviors, not observed with other forms of drinking.

Dendritic geometry is a key determinant of neurotransmission (Mainen and Sejnowski 1996; Krichmar et al., 2002; Vetter et al. 2001), and chronic alcohol exposure leads to changes in neuronal spine density and composition throughout the brain (Varodayan et al., 2018; Kim et al., 2015; Szumlinski et al., 2023; Holmes et al., 2012; Cuzon Carlson et al., 2011; Zhou et al., 2007). Our morphological analysis using diolistic labeling and dendritic spine analysis revealed significant decreases in total spine density in mPFC^{CRF1+} neurons following ethanol dependence. Considering that typically, potentiation of excitatory transmission correlates with an enlargement of spine size (Yuste and Majewska 2001; Alvarez et al. 2007), it was unexpected to observe reductions in spine density following ethanol dependence, especially in light of the observed increased glutamate release on mPFC^{CRF1+} neurons. Our data suggests that the heightened glutamate release might serve as a compensatory mechanism to augment glutamate transmission on mPFC^{CRF1+} neurons during dependence, despite the diminished spine density. Although the decreased spine density of mPFC^{CRF1+} neurons during dependence are driven by reductions in immature spine types including filopodia, long/thin spine types, and stubby spines. Importantly, even though the total spine density during withdrawal normalized to levels similar to those observed in naïve mice, the decreased count of stubby spines in mPFC^{CRF1+} neurons persisted throughout the withdrawal period. This was also observed in mPFC^{CRF1-} neurons, which did not undergo significant structural changes in total spine density during dependence and withdrawal but showed a selective decrease in stubby spines during withdrawal. Given that stubby spines are often associated with synapses that are undergoing remodeling or are in a state of synaptic plasticity, this suggests that chronic ethanol exposure may lead enduring rigidity of synaptic remodeling in the mPFC, consistent with decreased behavioral flexibility observed in individuals with AUD (Barker et al. 2014; Trick et al., 2014; Wolff et al., 2018; Fernandez et al., 2017; Gass et al., 2014a,b; Barker et al., 2017). Notably, these decreases in spine density mimic overserved effects of stress on mPFC prelimbic layer 2/3 neurons (Liu et al., 2021). Indeed, stress-induced CRF-CRF1 signaling results in mPFC and hippocampal dendritic atrophy underlying anxiety and memory deficits (Dong et al., 2018; Chen et al., 2010). Consistent with CRF's role in synaptic remodeling (Regev and Baram 2014), we found that mPFC^{CRF1+} neurons have reduced basal postsynaptic glutamatergic transmission, possibly due to ongoing basal CRF-CRF1 signaling regulating spine density. It is possible that alcohol dependence induces CRF release resulting in the dendritic remodeling as seen in other stress models. In fact, blocking CRF1 signaling during early life stress prevents stress-induced spine loss and PFC-related impairments in cognition (Liao et al., 2014). Together, this highlights reduced mPFC prelimbic synaptic spine density as potential neural substrate for negative affect seen in stress-related disorders such as alcohol dependence.

To better understand the enduring changes in mPFC^{CRF1+} neurons following ethanol dependence, particularly in line with the observed decrease in stubby spines during withdrawal, we investigated potential alterations in dendritic genes in mPFC^{CRF1+} during withdrawal. Our analysis revealed associations with neurotransmitter secretion, cytoskeleton dynamics, calcium signaling, and neuroinflammation. This is consistent with our previous study, where we demonstrated that withdrawal leads to increased expression of a hub gene encoding the neuroimmune mediator colony stimulating factor 1 (CSF1), which is important for the development and maintenance of microglia, in mPFC^{CRF1+} neurons (Patel et al., 2022). Previous studies have shown that stress-induced increases in mPFC CSF1 expression leads to microglia-mediated synaptic pruning underlying anxiety-like and depressive behaviors (Wohleb et al., 2018). This putative mechanism is consistent with the reduced stubby spine density we observed in mPFC^{CRF1+} neurons as well as the decreased postsynaptic glutamate transmission in this population we previously found (Patel et al., 2022). Indeed, the collective dysregulation of dendritic genes in mPFC^{CRF1+} neurons during withdrawal suggests a significant inflammatory response, potentially driving persistent structural and synaptic remodeling during withdrawal. The gene *Bgn*, responsible for producing biglycan—a structural component of the extracellular matrix and an activator of inflammasomes (Nastase et al. 2012)—and *HSD17B14*, which encodes a subtype of 17β-Hydroxysteroid dehydrogenases involved in regulating microglia-mediated inflammation (Saijo and Glass 2011), are upregulated in mPFC^{CRF1+} neurons. Conversely, *RNaseh2a*, which encodes ribonuclease H2 subunit A and is known to limit inflammatory gene expression (Sugawara et al., 2022), is downregulated

in mPFC^{CRF1+} neurons during withdrawal. Additionally, dysregulated dendritic genes suggest disruption of protein methylation processes (Al-Kachak and Maze 2023), a process not well studied in addiction field, which might disrupt neurotransmitter synthesis. Together, our analysis of dendritically associated genes in mPFC^{CRF1+} neurons indicate enhanced inflammation signals suggesting neuroimmune modulation which leads to the observed pruning and the electrophysiological changes, this model is supported by the dysregulation of extracellular matrix and cytoskeletal dendritic genes and the dysregulation of dendritic neurotransmitter related genes.

One limitation of our current work is that all experiments used male mice. There are significant sex differences in the PFC/mPFC glutamate system related to its release, receptors, and transporters, as well as its plasticity-related proteins (e.g. PSD95), with brain glutamate receptor levels fluctuating across estrous cycle (Giacometti et al., 2020; Knouse et al., 2022). It is not known whether similar sex differences exist in mPFC prelimbic layer 2/3 pyramidal neurons. Although our preliminary data suggests that mPFC^{CRF1+} glutamate transmission and ethanol induced decreases in glutamatergic transmission is similar in naive male and female mice (data not shown). In addition, we previously found a conserved role of mPFC^{CRF1+} neurons in regulating anxiety like behavior in both males and females (Patel et al., 2022). Together, this suggests that mPFC^{CRF1+} neurons may play a similar role in both males and females. However, given that females drink more alcohol and are faster to develop an alcohol dependence, the parameters for chronic intermittent vapor induction, used in this study, should be tailored to better model dependence and withdrawal in females. In addition, the known contribution of hormonal fluctuations contributing to alcohol drinking in females (Martin et al., 1999; Reid et al. 2003; Ford et al. 2002; Satta et al. 2018; Rodriguez et al., 2022; Kirson et al. 2018, 2021; Cruz et al., 2023; Khom et al., 2023) makes it difficult to directly compare males and females. For this reason, we focused the current study on male mice, which also enables cross-study comparison with our previous work (Patel et al., 2022). How mPFC^{CRF1+} neurons are dysregulated by ethanol dependence and withdrawal in females remains unknown. Despite this limitation, our study underscores the maladaptive changes in mPFC^{CRF1+} neurons following chronic alcohol exposure, highlighting the importance of investigating this population in female mice in future studies with an appropriate CIE model of alcohol dependence and withdrawal in females. The current findings support the further investigation of therapeutics that selectively target the aberrant excitation of mPFC^{CRF1+} population.

5. Conclusion

Overall, our study demonstrates the specific functional and morphological adaptations of the mPFC^{CRF1+} neuronal population represented by increased sensitivity in the glutamatergic transmission to acute and chronic ethanol exposure associated with changes in the spine density.

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CRediT authorship contribution statement

Reesha R. Patel: Writing – original draft, Visualization, Investigation, Funding acquisition, Formal analysis, Data curation. **Pauravi Gandhi:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kathryn Spencer:** Writing – review & editing, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Nihal A. Salem:** Writing – review & editing, Visualization, Software, Resources, Methodology, Formal analysis. **Chloe. M. Erikson:** Writing – review & editing, Investigation, Formal analysis. **Vittoria Borgonetti:** Writing – review & editing, Investigation, Formal analysis. **Roman Vlkolinsky:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Larry Rodriguez:** Writing – review & editing, Investigation, Formal analysis. **Tali Nadav:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Michal Bajo:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis. **Amanda J. Roberts:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Formal analysis. **R. Dayne Mayfield:** Writing – review & editing, Supervision, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Marisa Roberto:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2024.100657.

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