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Ethanol Alters Variability, but not Rate, of Firing in Medial Prefrontal Cortex Neurons of Awake, Behaving Rats.

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Abstract

Background: The medial prefrontal cortex (mPFC) is a brain region involved in the evaluation and selection of motivationally relevant outcomes. Neural activity in mPFC is altered following acute ethanol (EtOH) use and, in rodent models, doses as low as 0.75 g/kg yield cognitive deficits. Deficits in decision-making following acute EtOH are thought to be mediated, at least in part, by decreases in mPFC firing rates (FRs). However, the data leading to this conclusion have been generated exclusively in anesthetized rodents. The present study characterizes the effects of acute EtOH injections on mPFC neural activity in awake-behaving rodents.

Methods: Awake-behaving and anesthetized *in vivo* electrophysiological recordings were performed. We utilized three groups: the first received two saline injections, the second received a saline injection followed by 1.0 g/kg EtOH, the last received saline followed by 2 g/kg EtOH. One week later an anesthetized recording occurred where a saline injection was followed by an injection of 1.0 g/kg EtOH.

Results: The anesthetized condition showed robust decreases in neural activity and differences in up-down state dynamics. In the awake-behaving condition, FRs were grouped according to behavioral state: moving, not moving, and sleep. The differences in median FRs were found for each treatment and behavioral state combination. A FR decrease was only found in the 2.0 g/kg EtOH treatment during not moving states. However, robust decreases in FR variability were found across behavioral state in both the 1.0 g/kg and 2.0 g/kg EtOH treatment. Sleep was separately analyzed. EtOH modulated the up-down states during sleep producing decreases in FRs.

Conclusions: In conclusion, the changes in neural activity following EtOH administration in anesthetized animals are not conserved in awake-behaving animals. The most prominent difference following EtOH was a decrease in FR variability suggesting that acute EtOH may be affecting decision-making via this mechanism.

Keywords

ethanol; mPFC; anesthetized; awake-behaving; electrophysiology

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INTRODUCTION

The medial prefrontal cortex (mPFC) is a brain region involved in the evaluation and selection of motivationally relevant outcomes (Alexander, 2011; Euston, 2012; Tamura, 2017), which is hypothesized to facilitate top-down control over behavior (Franzen & Myers, 1973; Jahn, 2016; van Gaal, 2008; Tang, 2017; Rothschild, 2017; Gentry, 2018; Gutman, 2017). Previous data suggest that EtOH elicits robust decreases in mPFC neural activity, which may contribute to impairments in behavioral control observed following administration. These data, however, were obtained exclusively from anesthetized recordings performed in *in vivo* as well as *ex vivo* slice recordings (Tu et. al., 2007; Weitlauf, 2008; Badanich, 2013). Therefore, it is currently not known if EtOH produces similar decreases in mPFC activity in awake-behaving animals. Determining this is critical to better understanding of how alcohol, even at low doses, can lead to behavioral impairments.

Acute EtOH exposure produces deficits in cognition at doses as low as 0.75 g/kg (Givens, 1995; Ketchum, 2016; Chin, 2011). Cognitive effects are observed in the Morris Water Maze (Matthews 2000), radial arm maze (Matthews 2002), and contextual conditioned fear responses (Gulick, 2008; Hunt et. al., 2009; Kutlu, 2016, Van Skike, 2019). Additional cognitive deficits resulting from EtOH have been found in delay discounting (de Wit and Mitchell, 2010; Wilhelm, 2012) and novel object recognition (Ryabinin, 2002; Marszalek-Grabska, 2018). This broad effect on several different behaviors suggests that acute EtOH may impact multiple cognitive systems.

It has been hypothesized that a reduction in mPFC firing rates is responsible for the cognitive declines observed following acute EtOH. Tu et. al. (2007) showed a dose-dependent decrease in spontaneous mPFC activity following acute EtOH exposure in both anaesthetized *in vivo* recordings as well as in an *ex vivo* slice preparation. Importantly, these decreases were observed during cortical up-down states (UDS) *in vivo* and during current-clamp recordings within *ex vivo* slice preparations (Tu, et al., 2007). UDS are a state where neural dynamics alternate between hyperpolarized down-states, where little neural activity occurs, and depolarized up-states where a synchronous burst of activity occurs. Alterations in UDS dynamics were dependent on EtOH dosage and contributed to the net decrease in neural activity observed following EtOH administration. However, cortical UDS are only found naturally during deep sleep (Sanchez-Vives, 2010). Otherwise UDS are induced and modulated by the dosage of certain anesthetic agents or are found within *ex vivo* recordings (Dao Duc, 2015). Crucially, *ex vivo* slice preparations of mPFC cells exhibit similar UDS to *in vivo* anesthetized preparations (Tu et. al. 2007). EtOH has been found to decrease up-state amplitude, duration, and spiking frequency in a dose dependent manner (Tu, et. al. 2007). Furthermore, the alterations in up-state behavior were found to be NMDA receptor dependent (Weitlauf, 2008) and dependent on network-level changes induced during EtOH exposure (Woodward, 2009; Woodward, 2012). This suggests that NMDA receptor inhibition is a primary target of EtOH's actions in mPFC slice preparations and is congruent with findings in sensory cortices (Sessler, 1998).

Studies following chronic EtOH exposure also suggest that a primary action of EtOH on cortical tissue is inhibition of NMDA. Specifically, it is seen that long-term exposure to

EtOH enhances excitability in cortical areas suggestive of an adaptive response to ongoing EtOH induced inhibition (Nimitvilai, 2016; Cannady, 2018). Last, recent studies have investigated fast-spiking interneuron populations and have shown that chronic EtOH also alters their functionality (Hughes, 2020) suggesting that an overall change in inhibitory-excitatory (IE) balance develops over time and highlighting the need to investigate separable populations of neurons (i.e. pyramidal versus interneurons). However, it is unclear how the altered neural dynamics of *ex vivo/ex vivo* preparations can reliably be translated to how EtOH impacts the neural dynamics of awake-behaving states necessary for cognition, necessitating the present study.

Urethane is a common anesthetic choice for cortical electrophysiological recordings. It spares spontaneous cortical activity, is long lasting, and requires a single injection; however, it may have significant interactions with EtOH (Hara & Harris, 2002). Previous research has directly compared the effect of anesthesia on EtOH's actions in the cerebellum. When urethane anesthesia was used, a sharp decrease in cerebellar spontaneous neural activity was seen after EtOH exposure. However, for all other anesthetics tested, EtOH excited neural activity in the cerebellum (Siggins et. al., 1987). This may be due to pharmacological similarities between EtOH and urethane. Urethane has inhibitory actions on NMDA receptors while having excitatory actions on GABAergic receptors. Hara and Harris (2002) noted this and suggested that the anesthetic most similar to urethane is EtOH. Ultimately it is difficult to conclude from urethane anesthetized data that EtOH-induced decreases in firing rates are responsible for deficits in decision making given the synergistic potential in their pharmacology.

Existing data acquired *in vivo* suggest that the effects of EtOH observed in reduced preparations does not hold in behaving animals. Linsenbardt (2015) investigated mPFC activity in alcohol-preferring "P" versus Wistar rats in a Pavlovian alcohol consumption task and found no correlation between alcohol intake and gross firing rates in the mPFC. Electrochemical and microdialysis approaches also suggest that EtOH in awake-behaving animals may have different effects compared to those in anesthetized animals (Selim 1996; Mishra 2015). However, the acute effects of alcohol were not detectable in these studies as they were obscured by other mitigating factors such as consumption history, thus motivating the need for the current study.

The present study first sought to characterize the blood ethanol concentration (BEC) time course of intraperitoneally injected EtOH. We then designed a series of experiments to investigate if awake-behaving mPFC neural activity is reduced in the same manner as urethane anesthetized *in vivo* recordings. We hypothesized that EtOH would have little to no effect on neural activity in contrast to what has been seen in anesthetized preparations.

MATERIALS & METHODS

Animals

A total of 72 adult, male Wistar rats (Harlan – IN) with weights ranging from 300–450g and ages ranging from 3–4 months were used throughout this experiment. Importantly, all animals were completely EtOH naïve prior to receiving their respective treatments. 24

animals were utilized to measure the impact of acute EtOH injections on mPFC neural activity while the remaining 48 animals were utilized to characterize alcohol metabolism. All animals were single housed and kept on a reverse light cycle. All testing occurred during their dark cycle in red light. All experiments were carried out in accordance with procedures approved by Indiana University-Purdue University Indianapolis' (IUPUI) Institutional Animal Care and Use Committee (IACUC).

Apparatus

An opaque open field (40.64 cm × 40.64 cm × 38.1 cm) was used for all recordings. A Doric Commutator (Doric Lenses – Canada) was placed above the open field to allow the rat to explore and move freely. An OpenEphys (OpenEphys – MA) acquisition system was used to collect all electrophysiological data. AnyMaze (ANY-maze Behavioral tracking software – UK) was used to collect all behavioral and locomotor data.

Surgical Procedures

Animals were first anesthetized under isoflurane (2.5% at 4 L/h for induction, 2% at 1.0 L/h for maintenance). Fur was shaved and animals were placed in a stereotaxic device. Following sanitation of the incision area with 70% EtOH and betadine, the local anesthetic Marcaine was applied (5 mg/kg, s.c.). An incision was made to expose the skull, the skull was then cleaned, and bregma-lambda coordinates were identified. Stainless steel anchoring screws were inserted prior to probe implantation. A rectangular craniotomy was performed over the mPFC (AP: 2.8, ML: 0.3, DV: 3.0 from bregma). Following the craniotomy, the dura was removed, and the insertion site was cleaned using sterile saline. The probe was then lowered to the target site, cemented into place, and grounded to 2 screws located above the cerebellum. Ketoprofen (5 mg/kg, s.c.) and cefazolin (30 mg/kg, s.c.) were administered post-surgery and the animals recovered in a heated cage until recovery of their righting reflex.

Electrophysiological Equipment

32 channel microwire arrays were constructed using 23 μm diameter tungsten wires (California Fine Wire, CA), an in-house fabricated electronic interface board (EIB), and an in-house fabricated wire housing made of 100 μm diameter silicon tubing. A male Omnetics (Omnetics – MN) connector was soldered to a manufactured copper circuit board (PCB, design developed by the Likhtik Lab). The wire housing was a 16×2 array. Wires were fed through the wire housing and attached to our EIB. On experimental days, surgically implanted probes were connected to an Intan Omnetics headstage (Intan – CA). Data was collected via an OpenEphys acquisition box and sampled at 30 kHz. Locomotor data was concurrently collected in OpenEphys via AnyMaze's output.

Blood Ethanol Concentration Curve

A blood ethanol concentration (BEC) curve was generated using a cohort of 48 adult, male Wistars in both awake-behaving and anesthetized conditions. In the anesthetized condition, animals received a 1.5 g/kg of urethane. They then were given either a dose of 1.0 g/kg EtOH or 2.0 g/kg EtOH. A small incision was made at the tip of the animal's tail and 50 μL

of blood was sampled at 5, 15, 30, 60, and 90 minutes post-EtOH injection. Plasma was collected and frozen at −20C until analyzed using an Analox machine and Analox alcohol reagent kit (Analox Instruments Ltd. – UK).

Upon acquisition of BEC values, data was imported and analyzed in MATLAB. Equation 1was fit to the data using MATLAB's non-linear regression function fitnlm. This allowed us to reliably estimate BEC values for each EtOH dose throughout the entire time course of the electrophysiology recording. This informed recording length and data analysis.

$$Bec(t) = \left(1 + \ln\left(Be(t)^2\right) * p_1\right) + (1 + \ln\left(t^2 + B(t)\right) + (-p_2 * t + Bd(t)) \quad (1)$$

In Equation 1, p_1 and p_2 are parameters fit to the data utilizing MATLAB's fitnlm function. Variable *Be* is the exponential increase of the blood ethanol concentration in the initial time course. Variable *B* is the parabolic-like increase found after the initial exponential increase. Variable *Bd* is the linear decay of EtOH according to its 0-order kinetics (Cederbaum, 2012).

Awake Behaving Recordings

Three separate surgery cohorts were tested. Test days occurred at least 1 week following surgeries to implant MWA's (Figure 1B). All testing was conducted in red light. At the beginning of each test, the animal was weighed and doses for either saline, 1.0 g/kg EtOH, or 2.0 g/kg EtOH were calculated. The animal was then connected to the electrophysiological data acquisition system and allowed to habituate to the open-field and headstage for a period of 15 minutes. Following the 15-minute period, the animal was returned to its home cage and acclimated to the testing room for 4 hours. After 4 hours, animals were reconnected to the acquisition system and placed inside of the open-field and data acquisition was initiated (Figure 1C). A baseline recording period of at least 10 minutes was acquired. Following the baseline recording period, the animal received its first injection. For Groups 1 and 2, the saline injection was volume matched to a 1.0 g/kg dose of EtOH. For Group 3 the saline injection was volume matched to a 2.0 g/kg dose of EtOH. A period of 30 minutes followed this first injection and the animal was then given a second injection. For Group 1, the second injection was additional saline. For Group 2 the second injection was 1.0 g/kg of EtOH. For Group 3, the second injection was 2.0 g/kg of EtOH. Following the second injection, the recording continued for 2 hours. At the end of the recording, 100 μL of blood was drawn via tail snip.

Anesthetized Recordings

Anesthetized recordings took place at least 1 week after the awake-behaving recordings described above. Animals were weighed and doses for urethane (1.5 g/kg), saline, and EtOH (1.0 g/kg) were calculated. Animals were then anesthetized and connected to the acquisition system. After cessation of spinal reflexes, the recording period began. A baseline period of at least 10 minutes was collected prior to any experimental manipulations. Following the baseline period, a saline injection volume matched to 1.0 g/kg of EtOH was given and a period of 30 minutes post-saline was recorded. Following this 30-minute period, an EtOH dose of 1.0 g/kg was given to all animals. A recording period of at least 2 hours followed

this EtOH injection. Blood samples were taken at the conclusion of the recording period in order to assess BEC.

Histology

Following the anesthetized recording the brain was lesioned and animals were then transcardially perfused using a chilled 4% paraformaldehyde (PFA) solution in phosphate buffered saline (PBS) at a pH of 7.3–7.4. Brains were extracted and allowed to post-fix in 4% PFA overnight and then placed into a 30% sucrose solution in PBS. Brain sections were taken at 50 μm, placed onto gelatin-subbed slides, and allowed to dry overnight. The brain sections were then stained using a cresyl violet protocol and imaged at 10x. Electrode placements were compared against Paxinos and Watson's Rat Brain Atlas and superimposed on a section of the atlas.

Data Analysis

Behavioral state classification—Locomotor data was obtained by finding the Euclidian distance between time points (sampled at 30000 Hz). The Euclidian distance obtained was then transformed into 1-second bins by taking the cumulative sum of distance traveled each second. A threshold based on the standard deviation of the movement signal was set. When the movement signal exceeded this threshold at a given time point, that time point was labeled a moving behavioral state. Fourier transforms were applied to LFPs via overlapping 10 s windows at 1 s intervals. Delta power was extracted by multiplying the mean weight of the first principal component with the raw FFT LFP data. This allows for robust detection of slow wave sleep (SWS) (Watson, 2016). A threshold was set using the standard deviation of the weighted delta power spectrum. When delta power exceeded this threshold for a consecutive 60 second period and no movement was detected, the animal was considered sleeping. Time periods that were less than a consecutive 60 second period were considered quiet rest and not analyzed in the present study. Time points that met none of the above criteria (sleep or moving) were labeled not moving.

Spike train analyses—Spike sorting consisted of an unsupervised and then supervised phase. In the unsupervised phase, the raw extracellular signal was spike-sorted using SpyKING-Circus (Yger, 2018). Raw data was median referenced and then bandpass filtered at 600 and 6000 Hz. The threshold for each dataset was manually set by inspecting the data with SpyKING-Circus's GUI. For the supervised phase, the results of SpyKING-Circus were exported to the Python based manual curation GUI Phy (https://github.com/cortex-lab/phy). Within Phy, clusters exported from SpyKING-Circus were evaluated. Clusters with firing rates less than 0.01 Hz were not considered. From here, we qualitatively evaluated mean waveform quality by finding putative action potentials. Clusters with characteristic action potentials and v-shaped autocorrelograms were then marked as "good" in Phy and exported to MATLAB. We then quantitatively curated data via ISI violations. ISI violations were determined by finding instances where a putative spiking unit fired more than twice during a 2 ms interval. If a cluster's spiking repertoire contained more than 5% ISI violations, that cluster was then discarded. Within these analyses multi-unit activity (MUA) was not evaluated.

Following the supervised phase of clustering, well-isolated units were analyzed in MATLAB™. Baseline analyses were as follows: firing rates were calculated by counting the number of spikes that occurred per second and then placed into 1-min bins. A baseline value for each neuron was calculated from the median firing rate of the 5 minutes of activity prior to an injection. Percentage of baseline values were then calculated for each minute bin. Difference in firing rate analyses began by first placing data into 1-s bins. We then calculated the median firing rate per neuron from each behavioral state. A change score was then calculated between treatment epochs. Differences in variance proceeded similar to the above with the crucial difference being the calculation of the standard-deviation per neuron across the analyzed time-course. In percentage of baseline and change-score analyses, the first 30 minutes of activity following an injection was considered.

Up-Down State Analysis—Spike trains were binned at 10 ms and then spike train matrices were assessed via a Hidden Markov Model to detect periods of activity versus no activity across neural populations. Concurrent periods of activity were considered up states whereas inactivity are down states (Levenstein, 2019). For the anesthetized data, the entire time course was utilized and up-down state durations for saline and EtOH epochs were compared. For sleeping data, concurrent periods of sleep longer than 60 seconds were utilized. In addition, only periods of time following the second injection were used. For the saline-saline group, the full-time course following the second saline injection was utilized in order to obtain a sufficient sample of sleep states. For the saline-EtOH groups (EtOH 1.0 g/kg and EtOH 2.0 g/kg), only the first hour was utilized in order to capture EtOH specific effects without short term adaptations and metabolism. In order to minimize false positives in our up/down state detection, only up/down states (UDS) longer than 200 ms were analyzed.

Statistical Analyses—Data were checked for normality using a Kolmogorov-Smirnov test against the normal distribution except cases with less than 30 samples. All summary statistics with the exception of BEC data are presented as the median and the 95% confidence interval around the median. Firing rate distributions in all treatments and behavioral conditions were non-normally distributed. Data with three or more independent variables were first analyzed using the Kruskal-Wallis omnibus test. Significant omnibus tests were followed using Dunn-Sidak's test for multiple comparisons. In situations where 2 distributions were compared, either the Wilcoxon's Rank Sum test was utilized or the Wilcoxon's Sign Rank test if samples were dependent. All analyses were done in MATLAB.

Results

BECs following electrophysiology experiments do not differ from modeled BEC curve

A BEC curve was modeled to determine how blood alcohol levels would fluctuate during the electrophysiological experiments (Figure 2A). A curve was generated in a separate cohort of animals (e.g. no electrophysiology probes) receiving either 1.0 g/kg of acute EtOH or 2.0 g/kg of acute EtOH (i.p.) (Figure 2B). The model was capable of accurately predicting BEC values in both the 1.0 g/kg ($r^2 = 0.509$, $F(4,39) = 278$, $p < 0.001$) and 2.0 g/kg ($r^2 = 0.47$, $F(4,34) = 220$, $p < 0.001$) groups. Importantly, BECs collected at the conclusion of each

awake-behaving recording session did not significantly differ from our predicted values at the 120 minute timepoint (Figure 2B, 1 g/kg EtOH: $t(7) = 1.5$, $p = 0.17$. 2 g/kg EtOH: $t(8) = -0.26$, $p = 0.80$). This indicates that our modeled BEC curve is a good estimate of BECs throughout the recording period. Last, BEC values taken at the conclusions of the 1.0 g/kg Awake-Behaving recordings and the 1.0 g/kg Anesthetized recordings did not significantly differ (Figure 2C, $t(28) = -0.9$, $p = 0.33$).

Reduced neural firing in anesthetized animals is due to altered UDS

In urethane anesthetized animals, 1.0 g/kg EtOH produces a sharp decrease in neural activity in mPFC (Figure 3A, Friedman: $X^2 = 2152.73$, $p < 0.001$), thus our anesthetized results replicated previously reported data (Tu et. al., 2007). FDR corrected Wilcoxon Sign Rank tests indicate that the timecourse of this decrease is immediate and persistent. Additionally, the baseline corrected median firing rate of individual neuron's across the same timecourse shows a significant decrease in neural activity (Figure 3B, N = 623 neurons, $z = 46.9$, $p < 0.001$). Previous work with *ex vivo* slice preparations (Tu et. al., 2007) suggested that this decrease was attributable to changes in cortical UDS. Here, we replicate and extend these results as a decrease was observed during *in vivo* anesthetized recordings that is attributable to changes in UDS. Down state durations increase after EtOH injections (Figure 3B.3, Wilcoxon Rank Sum: $z = 14.69$, $p < 0.001$) while up state durations slightly decrease (Figure 3B.2, Wilcoxon Rank Sum: $z = -6.55$, $p < 0.001$).

No reductions were observed following EtOH administration in awake-behaving animals

The influence of alcohol on neural firing rates in mPFC was assessed in awake, behaving animals to determine if similar effects were observed to those the anesthetized animal. To control for the influence of repeated injections two saline injections separated by 30 minutes were given to a cohort of animals and no changes in firing rate were observed thus inidcating that repeated injections have no detectable influence on population firing rates (Figure 4A, Friedman: $X^2 = 0.92$, $p = 0.34$). Transient increases in FR were observed that were associated with the act of performing the injection and not attributable to EtOH, as they returned within 1–2 minutes to baseline post injection (Wilcoxon Signrank: $z = 0.11$, $p = 0.92$, timepoints −2 vs. 1 minute, first saline injection). In contrast to the anesthetized data, an overall reduction in firing rate was not detected in awake-behaving animals after receiving an injection of EtOH at 1.0 g/kg (Figure 4B, Friedman: $X^2 = 0.29$, $p = 0.59$). A main effect was found in the 2.0 g/kg EtOH dose (Figure 4C, Friedman: $X^2 = 37.76$, $p < 0.001$), however, a consistent decrease between individual saline and EtOH time points was not seen after FDR corrected Wilcoxon Sign Rank tests thus indicating an increase in variability of neural firing following injection. This increase in variance lead to the hypothesis that differences in behavioral states exist that may explain the variance in our EtOH injection data.

EtOH's Impact on mPFC Neural Activity is Dependent on State of Vigilance

To determine the influence of behavioral state on neural firing we split behavior into states of moving, not-moving, and sleep (Figure 5A). A 2-Factor ANOVA indicated that there was a main effect of time spent in each behavioral state (Figure 5B, ANOVA: $F(2,68) = 884.42$, $p < 0.001$) and indicated a significant interaction between behavioral state and the treatment

(Figure 5B, ANOVA: $F(4,68) = 22.01$, $p < 0.001$). The treatment by behavioral state interaction motivated us to probe further into the effects of how behavioral states may influence EtOH's effects on mPFC neural activity. Sleep states occurred late in the recording resulting in a lack of within-subject sleep measurements, therefore they were analyzed separately. An examination of the moving and not-moving behavioral states revealed that EtOH reduces neural activity in the non-moving condition, while no detecable changes were observed in the moving state (Figure 5C.2, Kruskal-Wallis: $X^2 = 35.43$, $p < 0.001$). Upon further analysis of this observation, we determined it was being driven primarily by decreases in the 2.0 g/kg EtOH NMV state (Figure 5C.2, Dunn-Sidak: 2.0 g/kg EtOH vs. 1.0 g/kg EtOH: CI: [42.7, 117.1], $p < 0.001$, 2.0 g/kg EtOH vs. Saline: CI: [38.68, 117.7], $p < 0.001$). This suggests that EtOH's effects observed in the awake-behaving animal are ultimately distinct from those observed in the anesthetized animal. More specifically, reductions in firing of mPFC neurons following EtOH injections in the awake behaving animal is likely attributable to EtOH's influence on vigilance state.

EtOH increased the variance amongst the population of neurons wherein several increased their firing rate and several decreased (Figure 5C.1, Figure 5C.2). Despite the increase in variance amongst the population of recorded neurons, the variance of an individual neuron's firing rate decreased after both 1.0g/kg and 2.0 g/kg of EtOH (Figure 5D). This effect was most pronounced in the moving condition (Figure 5D.1, Kruskal-Wallis: $X^2 = 66.8$, $p < 0.001$) where our 2.0 g/kg EtOH dose produced decreases in an individual neurons' variability when compared to both the saline (Dunn-Sidak: CI: [89.5,167.9], $p < 0.001$) and 1.0 g/kg EtOH conditions (Dunn-Sidak: CI: [49.0, 122.9], $p < 0.001$). A modest decrease in variance was also observed between the 1.0 g/kg EtOH treatment and saline (Dunn-Sidak: CI: [0.1, 85.3], $p = 0.049$). Modest decreases in variance were also found in the NMV state (Kruskal-Wallis: $X^2 = 24.79$, $p < 0.001$) where 2.0 g/kg EtOH produced decreases in variance compared to both the 1.0 g/kg EtOH (Dunn-Sidak: CI: [1.9, 75.8], $p = 0.036$) and saline (Dunn-Sidak: CI: [43.36, 121.81] $p < 0.001$) treatments. Additionally, the 1.0 g/kg EtOH treatment produced significant decreases in variance compared with saline (Dunn-Sidak: CI: [1.1, 86.31], $p = 0.043$).This suggests that EtOH is differentially affecting populations of neurons causing them to either increase or decrease in their firing rates. After this change occurs neural firing rates then become more stable.Ultimately EtOH is most consistently altering firing rate variance but not the firing rate itself.

Pyramidal Neurons Drive Differences in Firing Rate and Variability

Neurons were split into putative pyramidal and interneurons based on their mean waveform characteristics (peak to trough duration and time to polarization) based on a method from Ardid et. al. (2015). A Hartigan's dip test confirmed that the distributions of interneurons and pyramidal neurons were bimodal suggesting that the classification meaningfully separated the population (Figure 6A.2, Hartigan's Dip Test: $p < 0.001$). From there, similar analyses were ran on the classified dataset as were previously described above. EtOH at 2.0 g/kg produced no firing rate differences in the pyramidal-MV state (Figure 6B.1, Kruskal-Wallis: X^2 = 4.86, $p = 0.09$). EtOH at 2.0 g/kg did result in significant decreases in firing rate within the pyramidal-NMV state (Figure 6B.2, Kruskal-Wallis: $X^2 = 23.31$, $p < 0.001$) compared to saline (Dunn-Sidak: CI: [25.2, 90.9], $p < 0.001$) and the 1.0 g/kg EtOH

treatment (Dunn-Sidak: CI: [19.8, 81.1], $p < 0.001$). Qualitatively we see that within the interneuron populations, there is a small, non-significant decrease in the saline condition that is not apparent in either EtOH condition. Interneurons in both the MV (Figure 6B.3, Kruskal-Wallis: $X^2 = 5.18$, $p = 0.075$) and NMV (Figure 6B.4, Kruskal-Wallis: $X^2 = 1.08$, $p = 0.58$) behavioral states exhibited no changes in firing rate. Last, a reduction in firing rate variability was observed in the pyramidal-MV state following EtOH (Figure 6C.1, Kruskal-Wallis: $X^2 = 54.9$, $p < 0.001$). Post-hoc tests indicated that EtOH at both 1.0 g/kg (Dunn-Sidak: CI: [5.8, 77.5], $p = 0.02$) and 2.0 g/kg (Dunn-Sidak: CI: [68.0, 134.4], $p < 0.001$) produced decreases in pyramidal neuron's firing rate variability compared to our saline control. Additionally, a difference between the two EtOH treatments exists (Dunn-Sidak: CI: [28.6, 90.5], $p < 0.001$). In agreement with our unclassified data, a reduction in firing rate variability was also found within the pyramidal-NMV state (Figure 6C.2, Kruskal-Wallis: $X^2 = 22.2$, $p < 0.001$). This effect was driven by decreases in between both EtOH treatments and saline (Dunn-Sidak: EtOH 1.0 g/kg vs Saline: CI: [5.5, 77.1], $p = 0.02$. EtOH 2.0 g/kg vs Saline: CI: [33.4, 99.7], $p < 0.001$). Within our observed population, it is likely that EtOH is mostly impacting pyramidal neurons. However, the small sample of inteneurons warrants consideration.

Acute EtOH Disrupts UDS Dynamics during Sleep States

Neural activity was assesed within sleep epochs. During sleep, EtOH at 2.0 g/kg produced broad reductions in neural activity (Figure 7A, Kruskal-Wallis: $X^2 = 34.03$, $p < 0.001$) compared to saline treated sleeping animals (Dunn-Sidak: CI: [28.35, 69.67], $p < 0.001$) and animals receiving a dose of 1.0 g/kg EtOH (Dunn-Sidak: CI [36.16, 65.01], $p < 0.001$). Given that EtOH's reductions on neural activity in anesthetized animals occurs in the context of UDS, we hypothesized that the changes in non-anesthetized animals may also be due to disruptions in UDS dynamics. We found that up-state durations in both EtOH conditions were longer than in our saline condition (Figure 7B.2, Kruskal-Wallis: $X^2 = 79.55$, $p < 0.001$, Dunn-Sidak: Saline vs. EtOH 1 g/kg: $p < 0.001$, Saline vs. EtOH 2 g/kg: $p < 0.001$). Additionally, down-state durations were also longer compared to saline in both our EtOH 1.0 g/kg condition (Figure 7B.3, Dunn-Sidak: CI: [−3.90,−3.33], $p < 0.001$) and EtOH 2.0 g/kg condition (Dunn-Sidak: CI: [−1.12, −0.68], $p < 0.001$). The increase in down state duration is in agreement with what was found in anesthetized conditions, however, it was not a dose dependent increase given that the median down state duration after 2.0 g/kg of EtOH was less than the median down state duration after 1.0 g/kg of EtOH (Dunn-Sidak: [CI: 2.49, 2.94], $p < 0.001$). Interestingly, up state durations were consistently longer in both of our EtOH conditions which is an opposite effect of what is observed during anesthetized recordings. These results indicate that EtOH is decreasing neural activity within sleep states via disruption of UDS dynamics. However, the differences between up state durations in the awake vs the anesthetized recordings suggests differences in how EtOH infleunces UDS dynamics.

Discussion

Here we replicated previously reported data that shows mPFC neural activity is decreased in urethane anesthetized animals following a dose of 1.0 g/kg EtOH. The decrease in neural

activity is due to lengthening of down states and subsequent shortening of up states. We found that during sleep, EtOH may affect neural activity via disruption of UDS dynamics, however, this disruption is not entirely consistent with disruptions of UDS dynamics seen in our anesthetized preparation.

In order to further characterize the effects of EtOH on mPFC activity, we split behavior into three states: moving, not moving, and sleeping. From here, we found that the only instances in which neural activity is decreased *in vivo* is during periods of non-movement and sleep. Specifically, it was found that only the 2.0 g/kg dose of EtOH was sufficient to reduce neural activity during non-moving awake conditions. This decrease occurs during a reduced state of vigilance therefore it is not dissociable from the effects of EtOH on altered vigilance states. This leads to the conclusion that EtOH does not elicit reductions in mPFC neural firing in awake-behaving animals as seen in anesthetized preparations.

Finally, EtOH reliably reduced variability in individual neuron's firing rates, which was likely attributable to changes observed in pyramidal neurons. Since this was observed across all stages of vigilance and multiple doses, this provides a candidate mechanism for the deleterious effects of EtOH on cognitive funciton. However, it is important to note that the absence of effect in our interneuron population may be due to insufficient sample size.

Inconsistent results between anesthetized and awake-behaving recordings

To our knowledge, the present work is the first to look at the differential effects of EtOH on mPFC neural activity in anesthetized versus awake-behaving conditions. What we found was a significant departure from what has been reported previously in anesthetized *in vivo* recordings and *ex vivo* slice preparations and will inform future studies on EtOH's acute effects on cognition. However, the present work is not the first to investigate the differences between anesthetized conditions and awake-behaving conditions. For example, bistability is a general property of neurons that allows them to operate at multiple modes of firing and is reliably found in anesthetized (Loewenstein, 2005) but not awake-behaving preparations (Schonewille, 2006). Anesthesia has also been shown to influence sensory encoding by broadening the tuning curves of neurons when compared with awake-behaving recordings, thus indicating a blunting of stimulus specificity (Rinberg, 2006). These data highlight a few of the well-documented differences in brain activity during anesthetized and awake preparations.

Mechanisms of Up-Down States

At the microcircuit level, GABAergic mechanisms are hypothesized to maintain the balance between up and down states. GABA(A) receptor inhibition is necessary for regulating most aspects of UDS, while GABA(B) receptor inhibition has a more selective role in terminating up-states of layer 2/3 cortical neurons (Crunelli, 2015). Given EtOH's affinity for the GABA(A) receptor (Wallner et al., 2003) it can be hypothesized that layer 2/3 cortical neurons would be differentially impacted leading to differences in synchrony within and between brain regions. At the level of neurocircuitry, it has been shown that thalamocortical circuits are necessary for the production and maintenance of UDS (David, 2013; Lemieux, 2015). In instances where thalamocortical synchrony is decreased, deficits in memory

consolidation occur (Niknazar, 2015; Ladenbauer, 2017). In alcohol dependent patients, sleep-associated memory consolidation is impaired (Junghanns, 2009) and given EtOH's role in modulating up-down states (Tu et. al., 2007), it is tempting to speculate that the declines seen in memory consolidation in alcohol dependent patients may be due to a loss of thalamocortical synchrony. While this can partially explain the cognitive decline associated with long-term chronic, heavy alcohol use, it cannot fully explain the acute, pharmacological actions of alcohol on cognition.

Decreased firing rate variability may lead to impoverished encoding

In the present study, EtOH robustly decreased firing rate variability of individual neurons. Mechanistically, this may be due in part to reductions in NMDA conductance which has previously been reported to result in decreases in mPFC firing rate variability (Homayoun, 2004). Decreases in firing rate variability may have profound effects on mPFC-mediated behaviors given that the importance of neural variance in encoding evidence prior to decision making has been observed (Hussar, 2010; Churchland, 2011). Furthermore, it has been shown that variance in neural firing rates allows for flexible encoding of stimuli necessary for behavioral flexibility (Legenstein, 2014). EtOH-mediated increases in dopamine (Schier, 2013; Doherty, 2016), in concert with EtOH-mediated reduction of NMDA currents, may lead to reduced flexibility of encoding (Homayoun, 2004; Stalter, 2020). This may bias behavior towards the expression of learned or habitual behaviors and impair the ability to adaptively respond to dynamically changing environments. Reductions in behavioral flexibility have been observed following acute experimenter administered ethanol in both ethanol-naive (Matthews, 2002; Gawel, 2016) and ethanol-experienced rodents (Marszalek-Grabska, 2018).

Conclusions

Robust firing rate decreases are seen after EtOH treatment in the context of UDS, yet the same population of neurons yield drastically different responses in awake-behaving states wherein disruptions of firing rate variance are more prominent than reductions of firing rate. This highlights the importance of global brain dynamics on local responses to acute EtOH in the mPFC.

It is critical that future studies investigating how EtOH influences mPFC function identify which of the local effects of EtOH observed in awake-behaving *in vivo* preparations are also observable in *in vitro/ex vivo* preparations. This requires determining how *in vivo* mPFC neural activity and mPFC-mediated behaviors are influenced by EtOH in a concentration and temporally precise manner. Accomplishing this has the possibility to distill the numerous biophysical actions of EtOH to the most critical ones for impairments in cognitive function and AUDs.

An important caveat of the current study is that a single EtOH exposure was assessed, and while this is necessary to isolate the acute local effects of EtOH, linking these changes to those observed in AUD likely requires repeated EtOH exposures. In addition, in order to identify how EtOH's local effects on mPFC function influence computation in this brain region, experiments should be performed in behavioral tasks that engage mPFC. For

example, mPFC neurons exhibit alterations in firing rates as a function of task difficulty and distractors (Williams, 1999; Gill, 2000). Additionally, previous work has shown blunted firing rate increases during increased task difficulty in hippocampal neurons following EtOH (Givens, 1998). A similar mechanism may be found in the mPFC and warrants future investigation. In the current study the behavior was simple (exploration of an open field), and therefore the dynamics necessary to control behavior, and how they are influenced by EtOH, may not be detectable.

It is important to consider if the lack of correspondence between anesthetized and awake data generalizes to other brain regions. For instance, lateral septal neurons were found to not be disrupted by ethanol in either the anesthetized or awake-behaving cases while neighboring medial septal neurons were found to be inhibited in both conditions (Givens, 1989). This suggests that differences across brain regions could lead to differences in the susceptibility to acute EtOH and urethane. It is likely that granular cortical areas share similar responses to acute EtOH (Chapin, 1986; Chen, 2010) and also likely important to determine how the agranular structure of the rodent mPFC contributes to the present results (Laubach, 2018). Last, it is possible that the present effect does not generalize to adolescent rodents given that Van Skike et al. (2010) found reductions in cerebellar firing rate after acute EtOH only in adult rodents. Future work should determine whether organizational differences in granular and agranular cortical areas impacts acute EtOH response as well as determine whether the present results extend to adolescent rodents.

In summary, we found that effects of EtOH on neural activity in awake-behaving *in vivo* recordings are not congruent with those observed in *in vivo* anesthetized studies. The area of greatest convergence is during SWS, however, within SWS there exist differences in how acute EtOH alters UDS. The most robust effects of EtOH on *in vivo* neural activity were reductions in the variance of the firing rates and it will be critical for future studies to determine how this might influence computation in mPFC.

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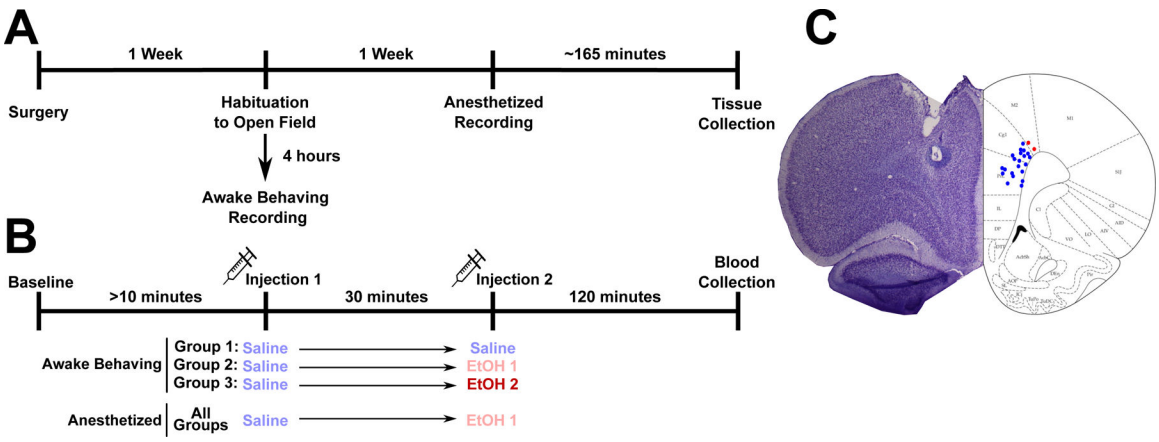

Figure 1. Experimental design and placements

A. Placements were confirmed via lesioning surrounding tissue prior to perfusing and extracting the brain. Placements were primarily located in the dorsalmedial prefrontal cortex. Specifically, these placements were primarily in the deep layers of the cingulate and prelimbic subregions. **B.** Animals underwent a single surgery as detailed in the Methods. Animals recovered for at least 1 week. Following the recovery period, animals were habituated to an open field where all subsequent recordings would take place. 4 hours after habituation, the awake-behaving recording took place for an average of 166 minutes. 1 week following the awake-behaving recording, animals were anesthetized with 1.5 g/kg urethane and neural recordings were performed. **C.** The recording period for both awake-behaving and anesthetized animals followed the same format. A baseline of at least 10 minutes was followed by administration of saline (i.p.). Following saline administration, animals were recorded for 30 minutes and then given their 2nd injection. In awake-behaving sessions the 2nd injection was either an additional dose of saline, 1.0 g/kg EtOH, or 2.0 g/kg EtOH. In anesthetized recordings, all animals received 1.0 g/kg EtOH. The recording would proceed for 2 hours after the second injection and at the cessation of recording blood samples were drawn to measure BECs.

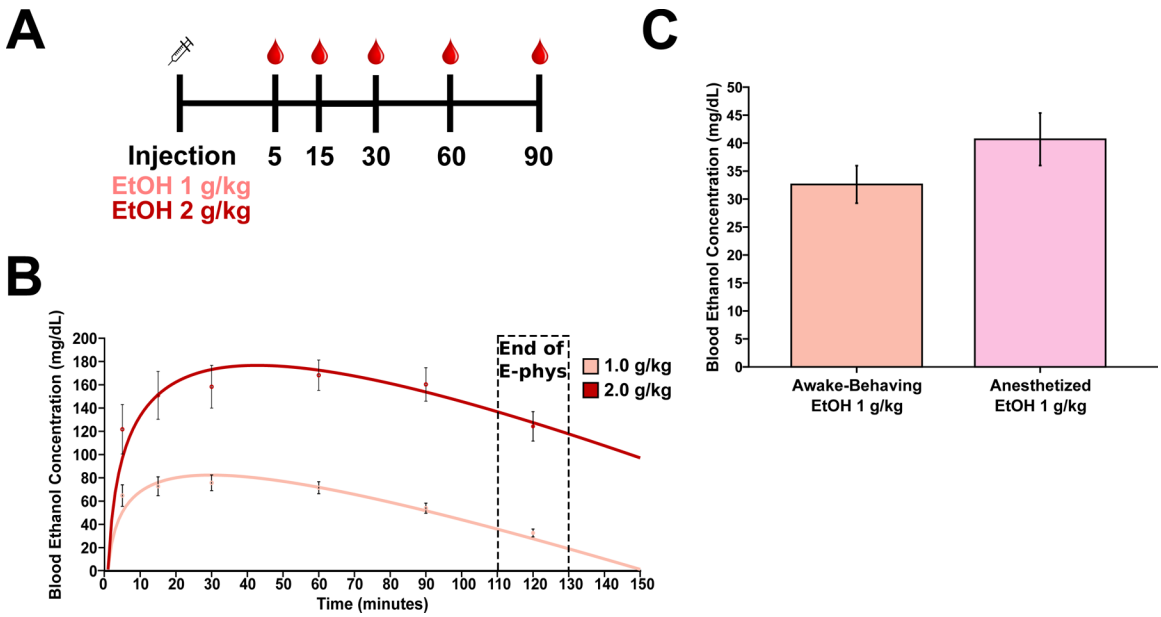

Figure 2. Modeled BEC Curve Informs Analysis

A. A cohort separate from our electrophysiology animals was used to model the time course of intraperitoneally injected EtOH in awake-behaving animals. An injection of either 1.0 g/kg or 2.0 g/kg was performed and then followed by tail-vein collection of blood at the +5, +15, +30, +60, and +90-minute marks. **B.** A parametrized equation generated our model BEC curve and was reliably fit to our empirical data for both our 1.0 g/kg dose ($r^2 = 0.509$, $F(4,39) = 278$, $p < 0.001$) and 2.0 g/kg dose ($r^2 = 0.47$, $F(4,34) = 220$, $p < 0.001$). Additionally, blood samples drawn at the end of our electrophysiology recordings did not significantly differ from our modeled BEC curve (One sample t-test, 1 g/kg EtOH: $t(7) = 1.5$, $p = 0.17$, 2 g/kg EtOH: $t(8) = -0.26$, $p = 0.80$). **C.** Awake-behaving BECs (N = 8) did not differ from anesthetized BECs (N = 20) in the 1.0 g/kg EtOH treatment (Two-tailed t-test, $t(28) = -0.9$, $p = 0.33$).

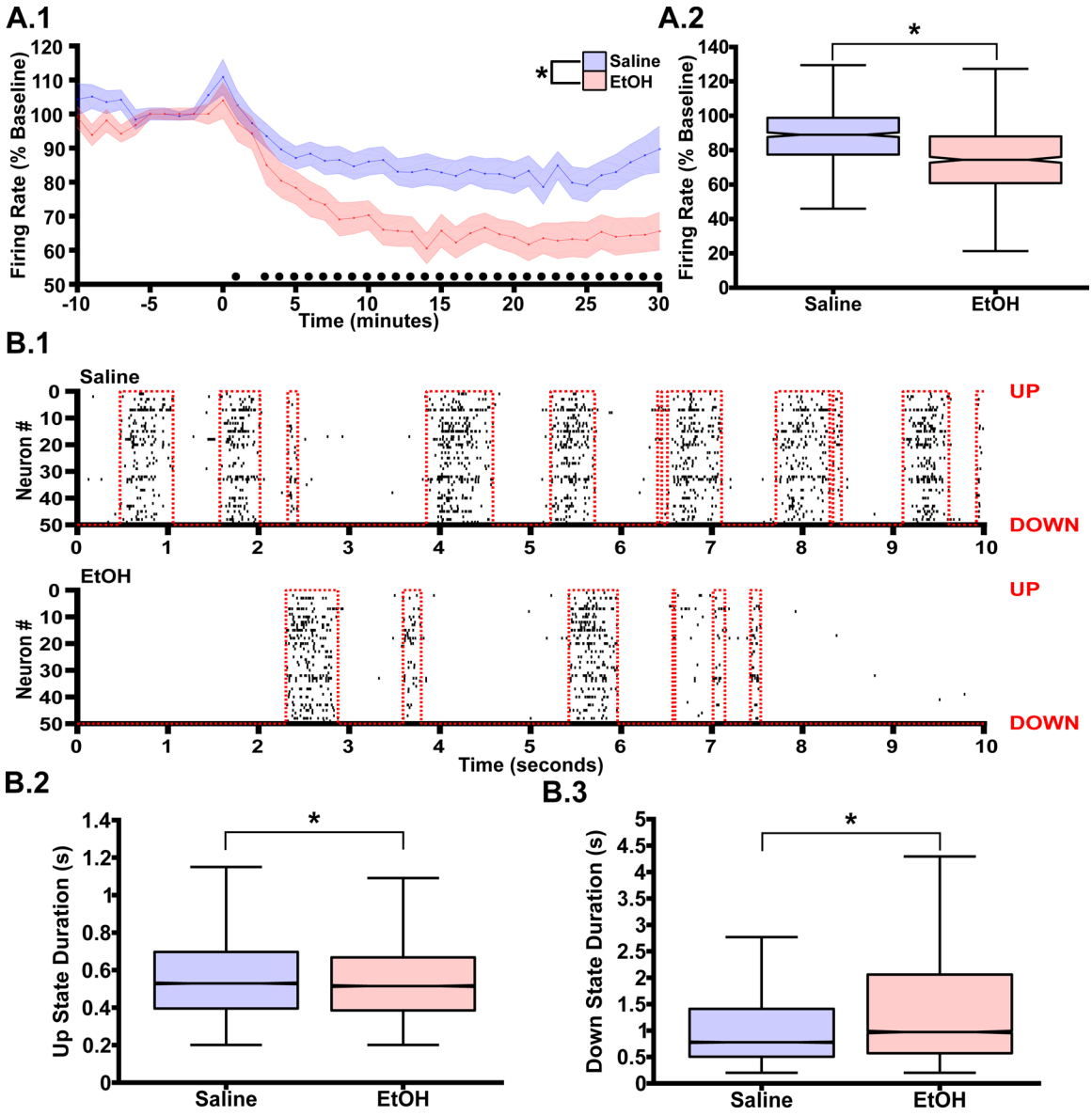

Figure 3. EtOH Produces Persistent Decreases in Anesthetized mPFC Neural Activity via Modulation of UDS.

A.1 The firing rate of our population of neurons was baseline normalized. The injection of either saline or 1.0 g/kg EtOH occurred at t=0 minutes. EtOH produced persistent decreases in firing rate (Friedman's ANOVA: $X^2 = 2152.73$, $p < 0.001$). Dark, black circles represent time points where neural activity between the two groups were significantly different. Data points represent the median firing rate while shaded error bars are the 95% confidence interval around the median. **A.2** The baseline normalized firing rate collapsed across time was also significant, suggesting a large portion of neurons reduced their median firing rate (N = 623 neurons, Wilcoxon's Rank Sum: $z = 46.9$, $p < 0.001$).

B.1 Up-down states were classified using a HMM process that identified points in time where the majority of neurons were either active or silent. The dotted red line represents the state (UP or DOWN). **B.2** EtOH significantly decreased the duration of up states

(Wilcoxon's Rank Sum: $z = -6.55$, $p < 0.001$, $N = 11,566$ for down states EtOH. $N = 16,117$ up states for saline). **B.3** EtOH significantly increased the duration of down states (Wilcoxon's Rank Sum: $z = 14.69$, $p < 0.001$, $N = 16,147$ down states for EtOH. $N = 21,121$ down states for saline).

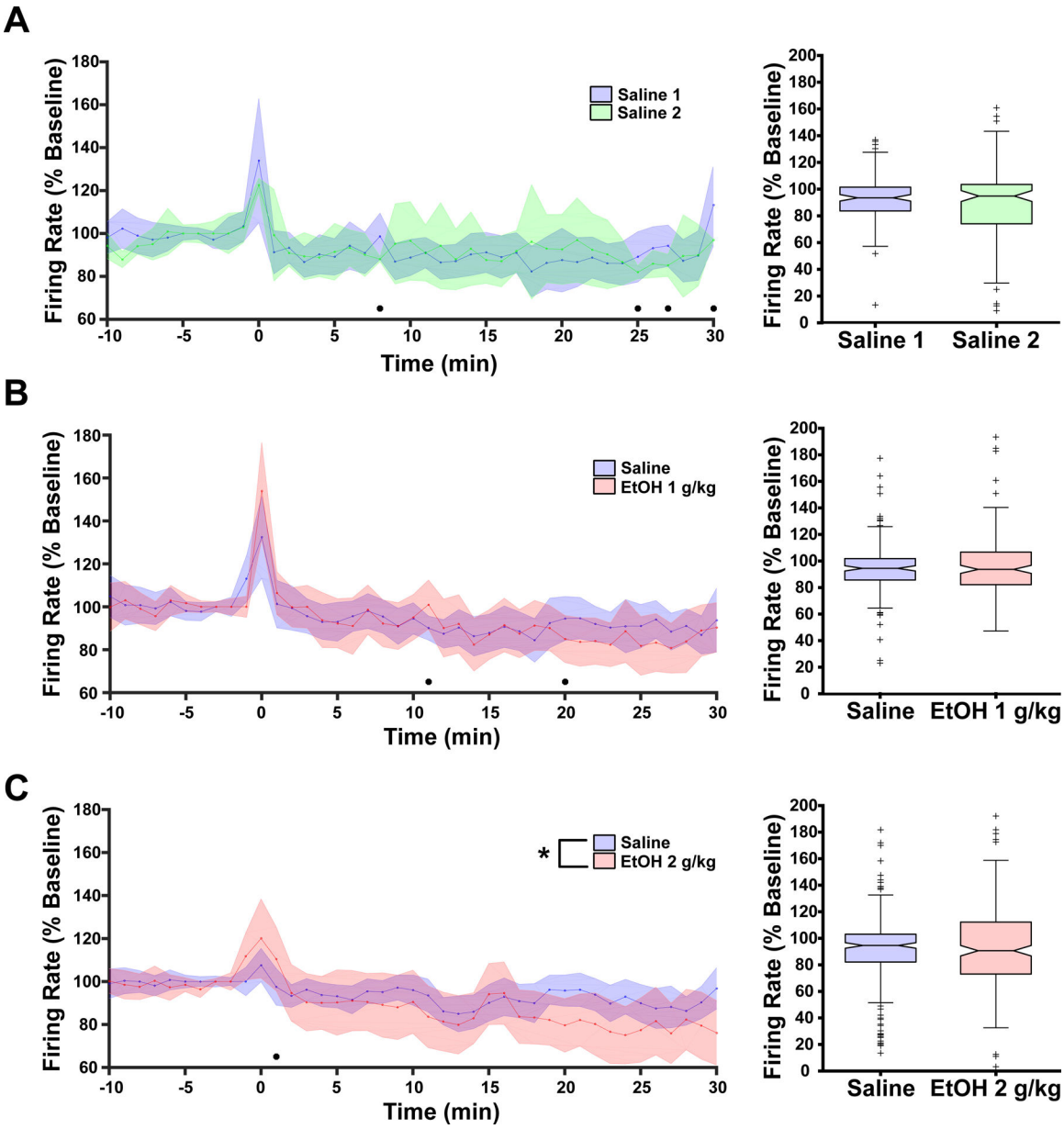

Figure 4. EtOH Does Not Produce Persistent Decreases in Awake-Behaving mPFC Neural Activity.

A. *Left.* The time course of two saline treatments is represented. Overall, no significant difference between the two groups was found (Friedman's ANOVA: $X^2 = 0.92$, $p = 0.34$, $N = 133$ neurons). *Right.* The median firing rate of the population of neurons shows no significant differences when collapsed across time (Rank Sum: $z = 1.33$, $p = 0.18$, $N = 133$ neurons). **B.** *Left.* The time course of a saline treatment followed by 1.0 g/kg EtOH. Overall, no significant difference between the two groups was found (Friedman's ANOVA: $X^2 = 0.29$, $p = 0.59$, $N = 161$ neurons). *Right.* The median firing rate of the population of neurons shows no significant difference when collapsed across time ($z = 0.22$, $p = 0.83$, $N = 161$ neurons). **C.** *Left.* The time course of a saline treatment followed by 2.0 g/kg EtOH. A significant difference was found between the two groups (Friedman's ANOVA: $X^2 = 37.76$,

$p < 0.001$, $N = 243$ neurons). However, no time points were found to be individually significant. *Right*. The median firing rate of the population of neurons shows no significant difference when collapsed across time (Wilcoxon Rank-Sum: $z = -0.02$, $p = 0.98$, $N = 243$ neurons). All data is represented as the median and 95% confidence interval around the median. • represents time points where an FDR corrected Wilcoxon Sign-Ranked test indicates significant difference between the two analyzed groups.

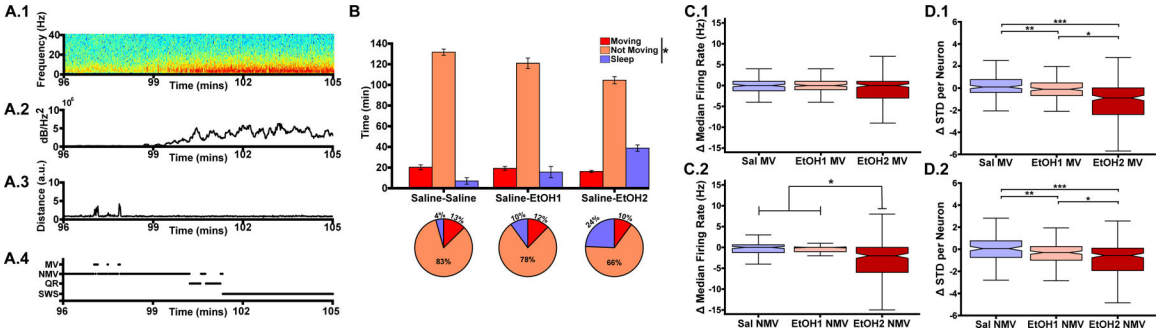

Figure 5. EtOH Decreases Firing Rate Variability

A. Description of behavioral state classification. **A.1.** A FFT-derived spectrogram illustrates time points where low-frequency power is dominant. **A.2.** Delta frequencies are extracted and a threshold is applied to produce putative time points where the animal is likely sleeping. **A.3.** Movement is calculated at a 1-s resolution. **A.4.** Behavior is categorized into one of 4 states: Movement (MV), No-Movement (NMV), Quiet Rest (QR), or Slow Wave Sleep (SWS). **B.** Based on the above categories, time spent in each behavioral state is assessed. A main effect of behavioral state was found (2-Factor ANOVA: $F(2,68) = 884.42$, $p < 0.001$) and a behavior by treatment interaction was found (2-Factor ANOVA: $F(4,68) = 22.01$, $p < 0.001$) which suggests that EtOH treatment modulates the amount of time spent sleeping. **C.** Firing rate differences per behavioral state and treatment. The difference in firing rate per neuron between 30-minute epochs after each injection is calculated. **C.1.** Firing rate differences within the MV state. No differences in firing rates were observed during states of MV (Kruskal-Wallis: $X^2 = 0.68$, $p = 0.684$). **C.2.** During states of NMV, 2.0 g/kg was found to produce a significant reduction in median firing rate (Kruskal-Wallis: $X^2 = 35.43$, $p < 0.001$) compared to 1.0 g/kg EtOH (Dunn-Sidak: CI: [42.7, 117.1], $p < 0.001$) and a significant reduction compared to saline (Dunn-Sidak: CI: [38.68, 117.66], $p < 0.001$). **D.** Differences in firing rate variability per behavioral state and treatment. **D.1.** During states of MV, variability of median firing rates of individual neurons in the 2.0 g/kg EtOH treatment exhibited a significant decrease (Kruskal-Wallis: $X^2 = 66.8$, $p < 0.001$) compared to both the saline (Dunn-Sidak: CI: [89.47, 167.92], $p < 0.001$) and 1.0 g/kg EtOH treatments (Dunn-Sidak: CI: [49.04, 122.94], $p < 0.001$). **D.2.** During states of NMV, a significant omnibus difference was found between all groups (Kruskal-Wallis: $X^2 = 24.79$, $p < 0.001$) and a significant difference between saline and the 2.0 g/kg EtOH treatment was found (******* Dunn-Sidak: CI: [43.36, 121.81], $p < 0.001$) as well as between the 1.0 g/kg EtOH treatment and saline (****** Dunn-Sidak: CI: [1.1, 86.31] $p = 0.043$). Last, a significant difference was found between the EtOH 2.0 g/kg and 1.0 g/kg treatments (***** Dunn-Sidak: CI: [1.93, 75.84], $p = 0.036$).

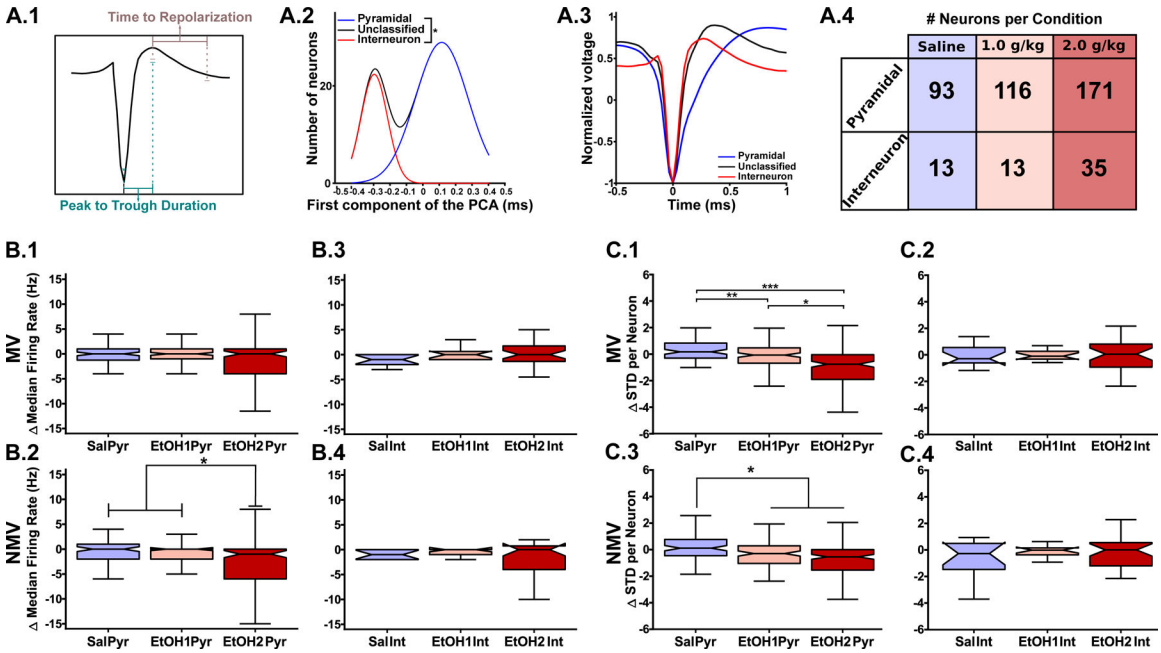

Figure 6. Decreases in Firing Rate and Variability Are Attributable to Pyramidal Neurons

A. Details and yield of waveform classification. **A.1.** Mean waveforms were processed by taking 4,000 4 ms samples. Mean waveform characteristics used for classification were the peak-to-trough duration and time to repolarization. **A.2.** The first principle component of the waveform characteristics was used to separate waveforms into broad, putative pyramidal neurons and narrow, putative interneurons. This classification produced a bimodal distribution (Hartigan's Dip Test: $p < 0.001$). Data that fell in between (unclassified, black line, N = 20) were not used in the present analysis. **A.3.** Mean waveforms of classified categories. **A.4.** Number of classified neurons per treatment and category. **B.** Differences in firing rate per treatment per behavioral state and per waveform classification. **B.1.** No differences were found within the pyramidal-MV groups (Kruskal-Wallis: $X^2 = 4.86$, $p = 0.088$). **B.2.** A significant reduction in firing rate was found in the pyramidal-NMV condition (Kruskal-Wallis: $X^2 = 23.31$, $p < 0.001$). Post-hoc tests indicated significant differences between the 2.0 g/kg EtOH pyramidal-NMV condition and the 1.0 g/kg EtOH pyramidal-NMV condition (Dunn-Sidak: CI: [19.82, 81.14], $p < 0.001$) as well as between the 2.0 g/kg EtOH pyramidal-NMV condition and the saline pyramidal-NMV condition (Dunn-Sidak: CI: [25.23, 90.82], $p < 0.001$). **B.3.** No differences were found within the interneuron-MV treatment conditions. (Kruskal-Wallis: $X^2 = 5.1$, $p = 0.078$). **B.4.** No differences were found within the interneuron-NMV condition (Kruskal-Wallis: $X^2 = 1.09$, $p = 0.58$). **C.** Differences in firing rate variability per treatment per behavioral state and per waveform classification. **C.1.** A significant reduction in firing rate variability (Kruskal-Wallis: $X^2 = 54.94$, $p < 0.001$) was observed in the EtOH 2.0 g/kg pyramidal-MV condition compared with the EtOH 1.0 g/kg pyramidal-MV condition (***** Dunn-Sidak: CI: [28.6, 90.5], $p < 0.001$) and the saline pyramidal-MV condition (******* Dunn-Sidak: CI: [68.03, 134.37], $p < 0.001$). Additionally, a significant difference was found between the EtOH 1.0 g/kg treatment and saline (****** Dunn-Sidak: CI: [5.85, 77.51], $p = 0.018$). **C.2.** A significant difference was observed in the pyramidal-NMV condition. (Kruskal-Wallis: $X^2 = 22.16$, $p <$

0.001). Post-hoc tests indicated a difference between the two EtOH treatment groups and saline (EtOH 1.0 g/kg vs. Saline: Dunn-Sidak: CI: [5.5, 77.1], $p = 0.02$. EtOH 2.0 g/kg vs. Saline: Dunn-Sidak: CI: [33.4, 99.7], $p < 0.001$). **C.3.** No differences were observed within the interneuron-MV treatment condition on firing rate variability (Kruskal-Wallis: $X^2 = 0.238$, $p = 0.888$). **C.4.** No differences were observed within the interneuron-NMV treatment condition on firing rate variability (Kruskal-Wallis: $X^2 = 1.311$, $p = 0.519$).

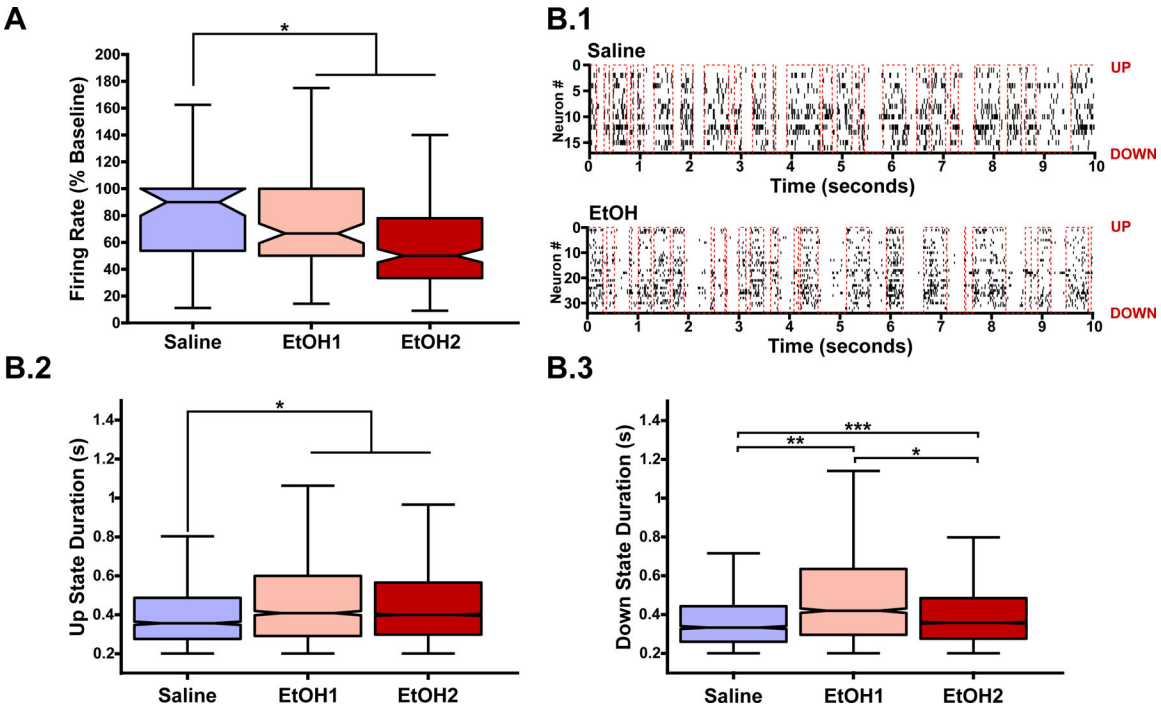

Figure 7. EtOH Reduces Neural Activity During Sleep via Disruption of UDS

A. EtOH treatment produced significant decreases in firing rate ($X^2 = 34.03$, $p < 0.001$) with post-hoc tests indicating that both the 1.0 g/kg EtOH treatment (Dunn-Sidak: CI: [36.16, 93.87], $p < 0.001$) and 2.0 g/kg EtOH (Dunn-Sidak: CI: [28.35, 110.83] $p < 0.001$) treatment produced significant decreases compared to the saline treatment. **B.1** Raster plots of representative up-down state classification per treatment conditions. **B.2** Up-state durations are increased following EtOH (Kruskal-Wallis: $X^2 = 79.55$, $p < 0.001$). Post-hoc tests indicate that both the 1.0 g/kg EtOH treatment (Dunn-Sidak: CI: [−987.62, −508.53], $p < 0.001$, N = 1972 up states for 2.0 g/kg) and the 2.0 g/kg EtOH treatment (Dunn-Sidak: CI: [−931.53, −535.00], $p < 0.001$, N = 6933 up states for 2.0 g/kg) show significant increases compared to the saline treatment (N = 1579). **B.3** Down state durations are also significantly increased following EtOH treatment (Kruskal-Wallis: $X^2 = 1000.16$, $p < 0.001$). Post-hoc tests indicate that the saline treatment (N = 2782 down states) is significantly different than both the 1.0 g/kg condition (Dunn-Sidak: CI: [−3,902, −3,329], $p < 0.001$, N = 2585 down states for 1.0 g/kg) and the 2.0 g/kg condition (Dunn-Sidak: CI: [−1,124, −675.4], $p < 0.001$, N = 10,126 down states for 2.0 g/kg).