Electrophysiological identification of daily rhythms in the prefrontal cortex

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Abstract

Circadian rhythms are ubiquitous in biology, from the molecular to behavioral levels. There is growing interest in understanding the functional implications of circadian oscillations in different cells and systems, including the brain. The prefrontal cortex (PFC) is heavily involved in myriad processes, including working memory, cognition, stress responses, and fear associated behaviors. Many PFC associated behaviors are time-of-day dependent, yet how time-of-day impacts the basic function of neurons in the PFC is not known. Here we use patch-clamp electrophysiology to record from layer 2/3 pyramidal neurons in the prelimbic (pl) PFC of male and female C57BL/6J mice at 4 separate bins of zeitgeber time (ZT): 0-4, 6-10, 12-16, and 18-22. We measured changes in membrane properties, inhibitory and excitatory inputs, ion channel function, and action potential kinetics. We demonstrate that the activity of plPFC neurons, their inhibitory inputs, and action potential dynamics are regulated by time-of-day. Further, we show that in males postsynaptic K^+ channels play a central role in mediating these rhythms, suggesting the potential for an intrinsic gating mechanism mediating information throughput. These key discoveries in PFC physiology demonstrate the importance of understanding how daily rhythms contribute to the mechanisms underlying the basic functions of PFC circuitry.

Introduction

Rhythms in life are found at many different time scales in nearly all phyla. From annual rhythms in hibernation and reproduction, to daily rhythms in sleep-wake cycles, to ultradian rhythms such as variations in heart rate, to rhythms in coordinated brain activity (Helm et al. 2013; Körtner and Geiser 2000; Yaniv and Lakatta 2015; M. H. Hastings, Reddy, and Maywood 2003). They are also present at nearly all levels of organization, from the behavior of groups of organisms to gene and protein expression at the cellular level (Jagannath et al. 2017; Landgraf et al. 2016; J. W. Hastings 2007). Given their ubiquity in nature and involvement in countless biological processes, understanding the functional significance of these rhythms is critical. However, while major strides have been made in understanding how these rhythms impact cellular function in the suprachiasmatic nucleus (SCN), as well as in some peripheral organs such as the liver, there remains a paucity of information about the functional impact of circadian clocks in other brain regions, beyond identifying that circadian rhythms are present (Abe et al. 2002; Albrecht and Stork 2017; Sato et al. 2020; Weaver 1998). This is a major gap in our knowledge, considering that circadian rhythms in behaviors are well documented, and shown to be critical in both health and disease.

The PFC serves as a critical component in cognition, emotional systems involved in fear learning and extinction, stress responses, and learning and memory, all of which are impacted by daily rhythms (Woodruff et al. 2018; McCarthy and Welsh 2012; Popoli et al. 2012; Sotres-Bayon, Cain, and LeDoux 2006; Miller and Cohen 2001). In addition, clock gene expression has been documented in the prefrontal cortex (PFC) (Chun et al. 2015). The prelimbic area (pl) of the PFC is divided into six distinct layers, each with distinct inputs and projections. Specifically, layer 2/3 plays a major role in working memory and behavioral plasticity and is involved in stress and depressive behaviors (Yuen et al. 2009; Zaitsev et al. 2012; Radnikow and Feldmeyer 2018; Moorman et al. 2015). The PFC is comprised of a wide array of cell types, including excitatory pyramidal neurons, which impact behavior by relaying information to other brain regions that are under clear circadian control, such as the amygdala and hippocampus, and inhibitory interneurons, such as the parvalbumin (PV+) and neuropeptide Y (NPY) containing neurons (Kawaguchi and Kubota 1997; Radnikow and Feldmeyer 2018; Vertes 2006; Saffari

et al. 2016). On whole, the function, synaptic inputs and intrinsic physiological characterizations of these neurons are heterogeneous throughout the PFC.

plPFC pyramidal neurons receive excitatory glutamatergic and inhibitory GABAergic presynaptic input, with findings suggesting these are differentially regulated in male and female mice (Popoli et al. 2012; Saffari et al. 2016; Andrade et al. 2012; de Velasco et al. 2015; Pena-Bravo et al. 2019). Excitatory inputs onto these neurons are plastic and environmental factors such as stress, learning and memory, can lead to long-lasting potentiation of glutamatergic inputs onto these neurons through increased NMDAR and AMPAR mediated currents (Yuen et al. 2009; Laroche, Jay, and Thierry 1990). Over activation of plPFC neurons is detrimental to normal behavioral function, and inhibitory inputs, which arise from the numerous inhibitory interneurons throughout the PFC, serve to mitigate the excitability of PFC neurons (Ferguson and Gao 2018). The majority of PFC pyramidal neurons are intrinsically quiescent at rest and regulate information throughput via a wide array of ion channels, including cyclic-nucleotide-gate non-selective cation (HCN) channels, and calcium (Ca^{2+}) and potassium (K^+) channels known to mediate postsynaptic throughput of excitatory and inhibitory currents (Kalmbach and Brager 2020; Zaitsev et al. 2012; Deng et al. 2019; Workman et al. 2015). In the SCN, changes in sodium (Na^+), K^+, and Ca^{2+} ion channel function mediate daily rhythms in the spontaneous activity, and action potential dynamics of neurons (Bano-Otalora et al. 2021). How these channels might impact daily rhythms in PFC function and the gating of information throughput is unknown.

Given the importance of understanding how daily rhythms impact PFC function, and our previously documented effects of circadian desynchronization on PFC structure (Karatsoreos et al. 2011), here we rigorously tested how time-of-day alters a wide range of neurophysiological properties in plPFC pyramidal neurons. The data presented here thoroughly demonstrate that time-of-day clearly impacts the basal activity of these neurons. Second, we show that inhibitory and excitatory synaptic inputs fluctuate throughout the day in a sex dependent manner. Lastly, we identify that K^+ channels may serve, in part, as a mechanism to regulate daily changes in information throughput in plPFC pyramidal neurons.

Results

Resting membrane potential of prelimbic layer 2/3 pyramidal neurons is rhythmic in male mice.

The regional and cell specific heterogeneity in electrophysiological properties of PFC pyramidal neurons has been described in multiple species (Zaitsev et al. 2012; van Aerde and Feldmeyer 2015; Piette et al. 2021). Layer 2/3 pyramidal neurons of the plPFC were identified visually by anatomical location (**Figure 1A;** *left*). Pyramidal neurons were identified by shape and lucifer yellow (LY; 0.2%) was added to the patch pipette for confirmation of an apical dendrite (**Figure 1A;** *right*).

To test our hypothesis that time-of-day impacts the basal electrophysiological properties of pyramidal neurons, we performed whole-cell patch clamp techniques and measured RMP, membrane capacitance (Cm), and membrane resistance (Rm) at ZT bins: 0-4, 6-10, 12-16, and 18-22 in male and female mice (**Figure 1B-E**). For RMP there was a main effect of time (no effect of sex) and interestingly, within group post-hoc analysis revealed that plPFC pyramidal neurons in male mice are more depolarized at ZT6-10 (light period), when compared to 12-16 and 18-22 (dark period). Post-hoc analysis did not reveal a time-of-day effect on RMP in female mice (**Figure 1C**). We did not observe an interaction between sex and time in any of our measures; however, there was a main effect of sex on Rm (**Figure 1E**). Together, these data demonstrate that the RMP of plPFC pyramidal neurons in male mice changes throughout the light/dark (LD) cycle.

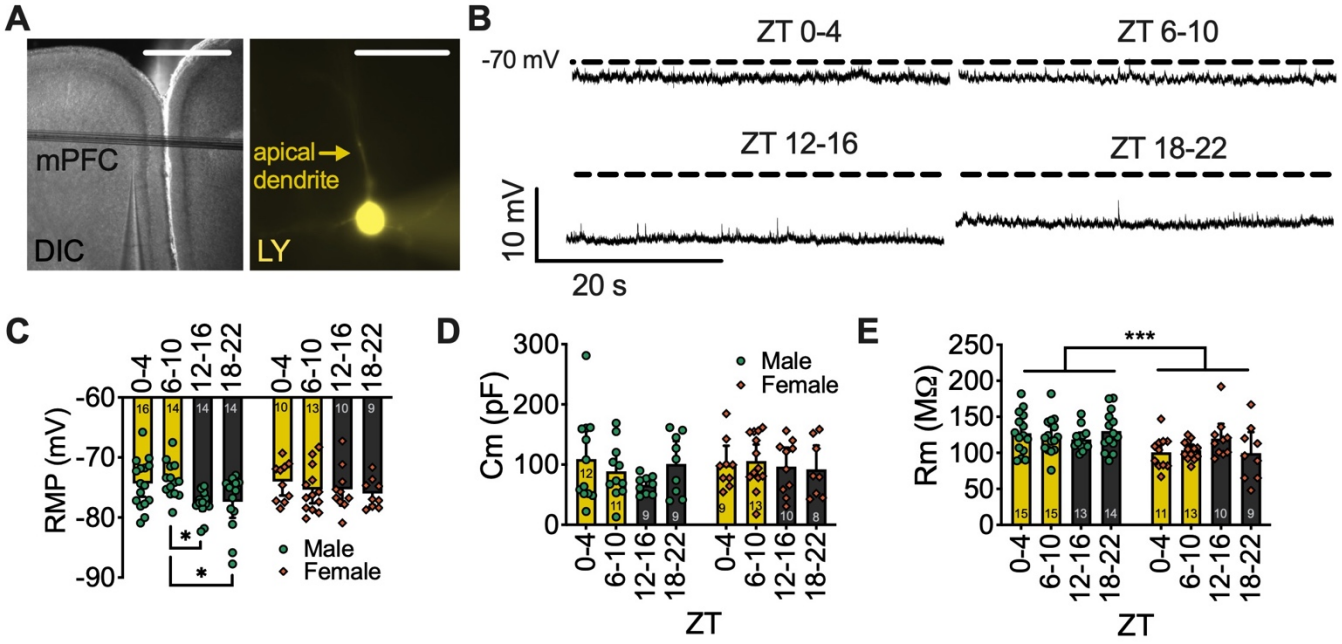

Figure 1. *Time-of-day changes in membrane potential of layer 2/3 mPFC pyramidal neurons.* **(A)** Image of mPFC slice (*left;* scale 1mM) and layer 2/3 pyramidal neuron backfilled with lucifer yellow (LY, *right;* scale 10 μM). **(B)** Representative traces of current clamp recordings from male mice at each ZT bin. **(C)** Mean and individual data points for membrane potential (RMP) at ZT0-4, 6-10, 12-16, and 18-22 in male (*bluish green circles*) and female (*vermillian diamonds*) mice **(D)** Mean membrane capacitance (Cm) and **(E)** resistance (Rm) binned by ZT. Error bars represent ± 95% CI. N-values for number of cells inset on bars. Two-way ANOVA for main effects and interaction with a within group Tukey post-hoc analysis for ZT bin, * $p < 0.05$, ***$p < 0.001$. Exact p-values, mouse N-values, and analysis in **Figure 1 – source data 1**.

sEPSC activity on plPFC pyramidal neurons is time-of-day dependent

Glutamatergic pyramidal neurons are the predominant cell-type in the plPFC, project to extra-PFC cortical, subcortical and limbic regions, and interconnect within the PFC (Le Merre, Ährlund-Richter, and Carlén 2021). We hypothesized that basal excitatory glutamatergic release contributes to daily changes in RMP and predicted that the number and/or strength of excitatory inputs are highest during the light period, when plPFC pyramidal neurons are depolarized (**Figure 1B, C**). To test whether time-of-day alters excitatory inputs we used the whole-cell voltage-clamp configuration (V_H = -70mV) to record sEPSCs in plPFC pyramidal neurons from male and female mice at ZT0-4, 6-10, 12-16, and 18-22 (**Figure 2A-E**). There was a main effect of ZT time on sEPSC frequency, but not amplitude, and post-hoc analysis demonstrated that in male mice the frequency of excitatory inputs was increased during the dark period, when RMP is hyperpolarized, an effect counter to our hypothesis (**Figure 1B and 2A-C**). Further, we observed clear sex differences on both sEPSC frequency and amplitude (**Figure 2D, E**). These data suggest that time-of-day impacts the number of excitatory inputs (frequency), but not their strength (amplitude) in male and female mice.

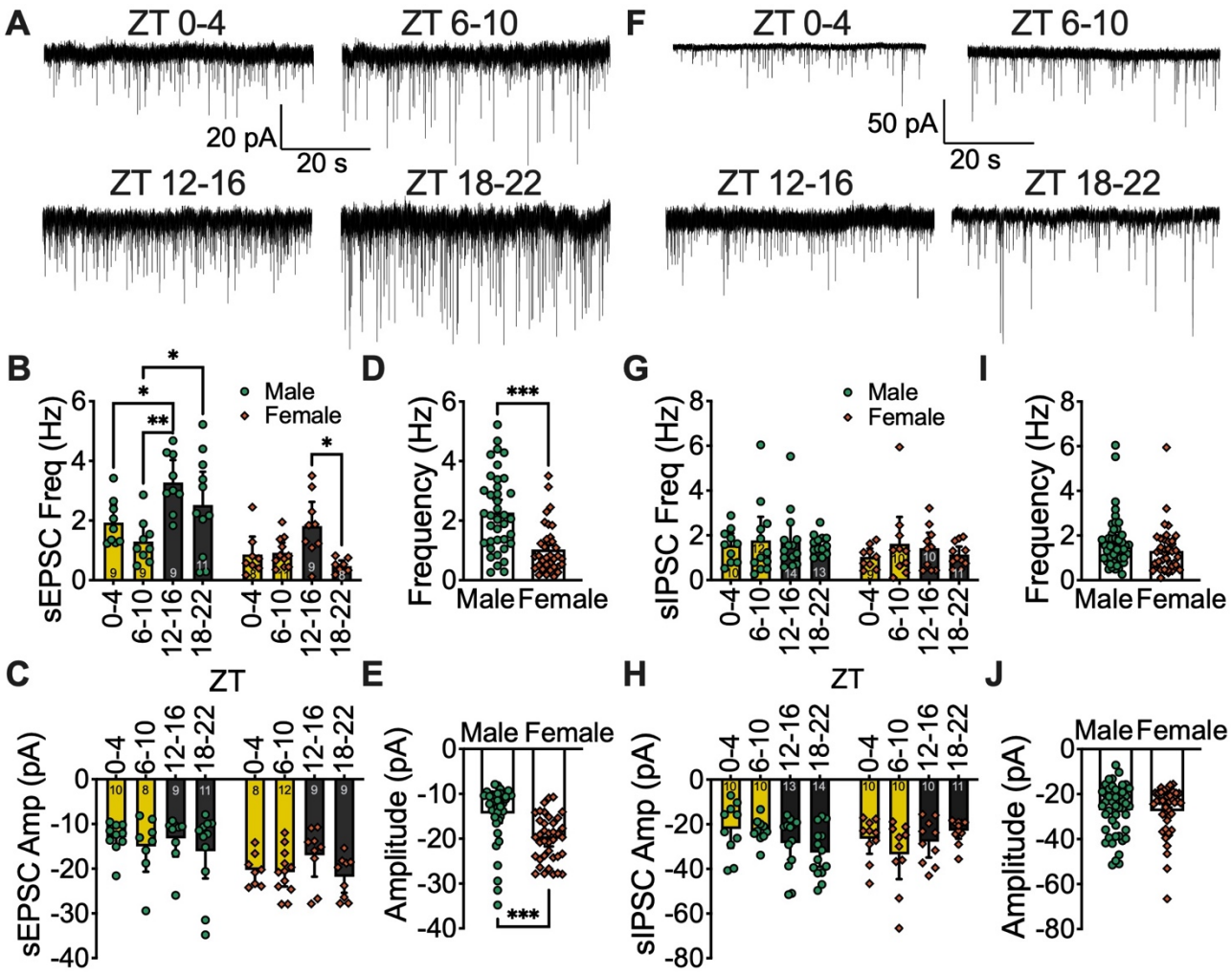

Figure 2. *Excitatory synaptic inputs onto plPFC pyramidal neurons are time-of-day dependent and differ by sex.* **(A)** Representative traces of sEPSC voltage clamp recordings from male mice at each ZT bin. **(B, D)** Mean and individual data points for sEPSC frequency and **(C, E)** amplitude at ZT0-4, 6-10, 12-16, and 18-22 or combined (*respectively*) in male (*bluish green circles*) and female (*vermillian diamonds*). **(F)** Representative traces of sIPSC voltage clamp recordings from male mice at each ZT bin. **(G, I)** Mean and individual data points for sIPSC frequency and **(H, J)** amplitude at ZT0-4, 6-10, 12-16, and 18-22 or combined (*respectively*) in male and female. Error bars represent ± 95%CI. N-values for number of cells inset on bars. Two-way ANOVA for main effects (including ***D, E, I and J***) and interaction, with a within group Tukey post-hoc analysis for ZT bin, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Exact p-values and analysis in **Figure 2 – source data 1**.

Time-of-day does not alter sIPSCs in male or female mice

Changes in RMP can be a consequence of intrinsic and/or synaptic properties. Our results on sEPSC frequency and amplitude (**Figure 2A-E**) in the plPFC suggest that excitatory synaptic inputs are not the primary driver of the daily changes in RMP illustrated in **Figure 1** and required further investigation to identify the mechanism underlying time-of-day changes in resting state. To investigate the contrast between daily changes in RMP and excitatory inputs, we predicted that if synaptic signaling is a primary mediator of time-of-day changes in RMP then inhibitory inputs should be robust and highest during the dark period ZT bins. GABAergic interneurons make up a small portion of PFC neurons, but are highly involved in the regulation of pyramidal neurons and relay information between different regions within the PFC (Saffari et al. 2016). To determine if inhibitory inputs contribute to the diurnal tone of pyramidal neurons, we recorded (s) inhibitory postsynaptic currents (IPSCs; V_{Hold} = -70mV) using a cesium chloride internal solution at ZT 0-4, 6-10, 12-16, and 18-22 (**Figure 2F-H**). We did not observe a significant difference in sIPSC frequency or amplitude in male or female mice (**Figure 2F-J**), but noted a trend in increased amplitude during the dark period in male mice (**Figure 2F, H**). Overall, these data do not support the notion that spontaneous synaptic inputs are the primary regulator of daily rhythms in neuronal resting state in plPFC pyramidal neurons, suggesting that a postsynaptic mechanism may regulate daily rhythms in these neurons.

mIPSCs onto plPFC pyramidal neurons are time-of-day dependent

Our central goal was to identify the mechanism(s) by which daily rhythms impact information throughput of plPFC pyramidal neurons. Given that we did not observe daily changes of RMP in female mice, and there was a main effect of sex on membrane resistance (**Figure 1C, E**), sEPSC frequency, and amplitude (**Figure 2D, E**) – all without an interaction between sex and ZT bin – we proceeded with our mechanistic investigation by separating male and females into independent groups.

Although we did not observe an effect of ZT bin on sIPSC frequency or amplitude in male mice, when ZT bins were combined into light period and dark period, we uncovered a significant impact of the LD cycle on sIPSC amplitude (**Figure 3B**). To further investigate whether time-of-day impacts the

presynaptic or postsynaptic components of inhibitory inputs, we measured mIPSCs by bath applying the voltage-gated sodium channel blocker tetrodotoxin, which isolates the synapse from upstream activity by inhibiting action potential firing. We observed no effect on mIPSC frequency, which is typically associated with presynaptic neurotransmitter release, in male mice (**Figure 3A, C**). Although we did not observe changes in decay tau or holding current, the time-of-day effect on IPSC amplitude persisted in this configuration, and this increase in amplitude of inhibitory inputs is consistent with the hyperpolarized RMP we observed during the dark period in male mice (**Figure 1C** and **3A, D-F**). Notably, in female mice there was no LD effect on sIPSC amplitude (**Figure 3H**), but we did observe an increase in mIPSC frequency and amplitude during the latter part of the light period (ZT 6-10; **Figure 3G, I-J**) although there was no effect on other potential postsynaptic measures, such as decay tau and holding current (**Figure 3K, L**).

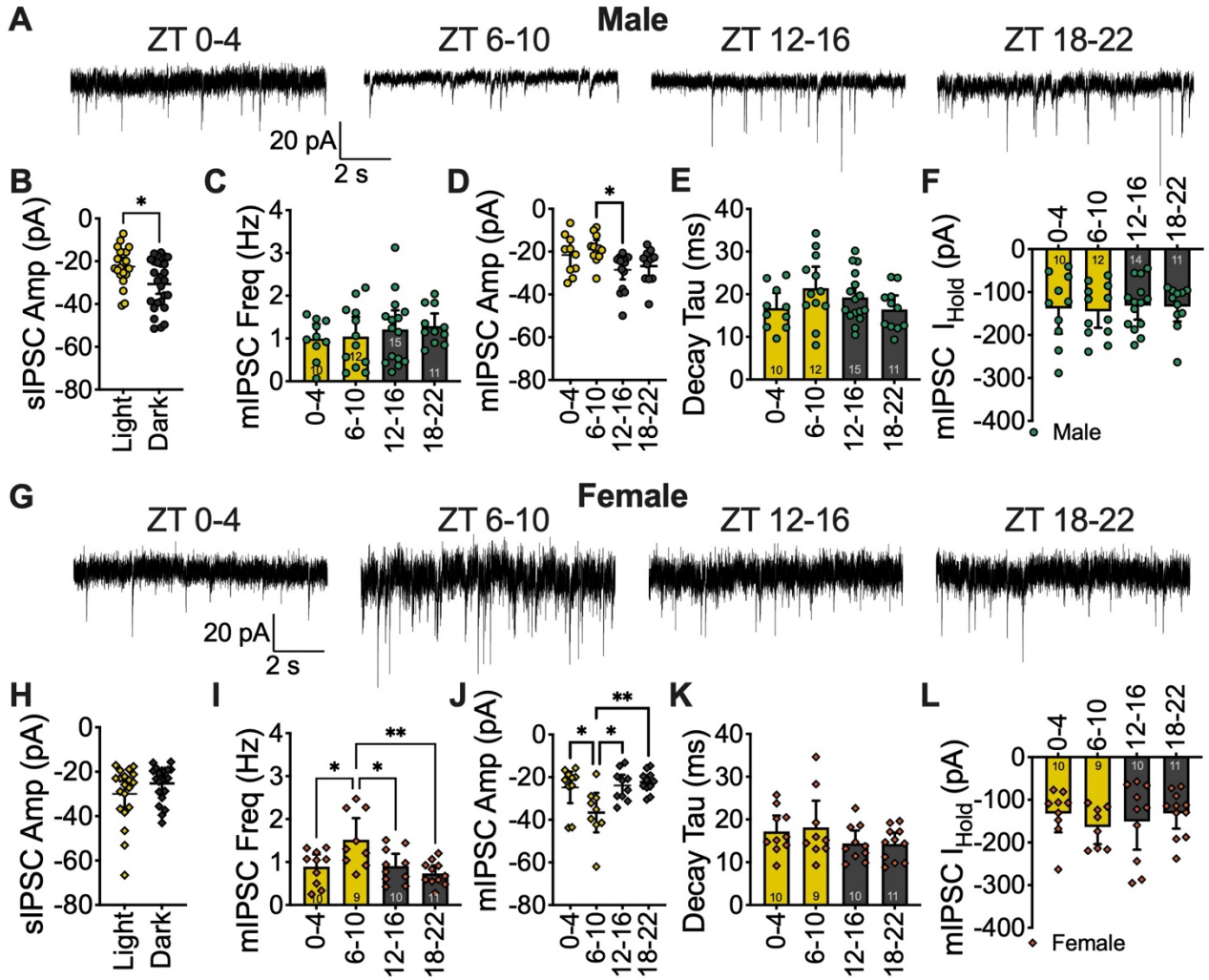

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Figure 3. *Strength of inhibitory synaptic inputs onto plPFC pyramidal neurons are time-of-day dependent in male and female mice.* **(A)** Representative traces of mIPSC voltage clamp recordings from male and **(G)** female mice at each ZT bin. **(B, H)** Mean sIPSC amplitude in LD cycle (unpaired student t-test; yellow fill = light period, charcoal fill = dark period) and **(C, I)** mIPSC frequency, **(D, J)** amplitude, **(E, K)** decay tau, and **(F, L)** holding current (I_{Hold}) at ZT0-4, 6-10, 12-16, and 18-22 in male (*circles*) and female (*diamonds*) mice. Error bars represent ± 95%CI. One-way ANOVA with Tukey post-hoc analysis for ZT bin, $*p < 0.05$, $**p < 0.01$. N-values for number of cells inset on bars, exact p-values, mouse N-values, and analysis in **Figure 3 – source data 1**.

Time-of-day impact on current voltage relationship in plPFC pyramidal neurons of female mice

Although mIPSCs were enhanced during the latter portion of the light period (ZT6-10) in female mice, we had not observed any convincing evidence of cell endogenous daily rhythms in the physiology of plPFC pyramidal neurons in these mice. To investigate any potential daily changes in postsynaptic properties we used a potassium (K^+) gluconate internal solution and measured the current-voltage (I-V) relationship in these neurons by performing a voltage-step inactivation protocol in which neurons held at -70mV were depolarized to 30mV and hyperpolarized in 10mV steps to a final MP of -120mV (**Figure 4A, B**). We analyzed the delayed steady-state current density (current density = $(I_{Total})/(Cm)$) during the hyperpolarized (from -120 mV to -70mV (*K1*); **Figure 4A-C**) and depolarized state (0 mV to 30mV (*K2*); **Figure 4A, B, E**), as well as daily changes in normalized conductance (g) calculated as the slope of the K1 and K2 steady-state current normalized to cell capacitance: $g_{Normalized} = ((I_{VH2} – I_{VH1}) / (V_H2 – V_H1))/(Cm)$, at different ZT bins (**Figure 4D, F**). We observed a main time-of-day effect on current density and normalized conductance for the K1 hyperpolarized voltage steps, although there was no main effect at the K2 depolarized voltage steps (**Figure 4B-F**). Together, these data demonstrate that female mice do display daily rhythms in postsynaptic membrane properties, but they are not robust enough to alter resting state (**Figure 1C-E**).

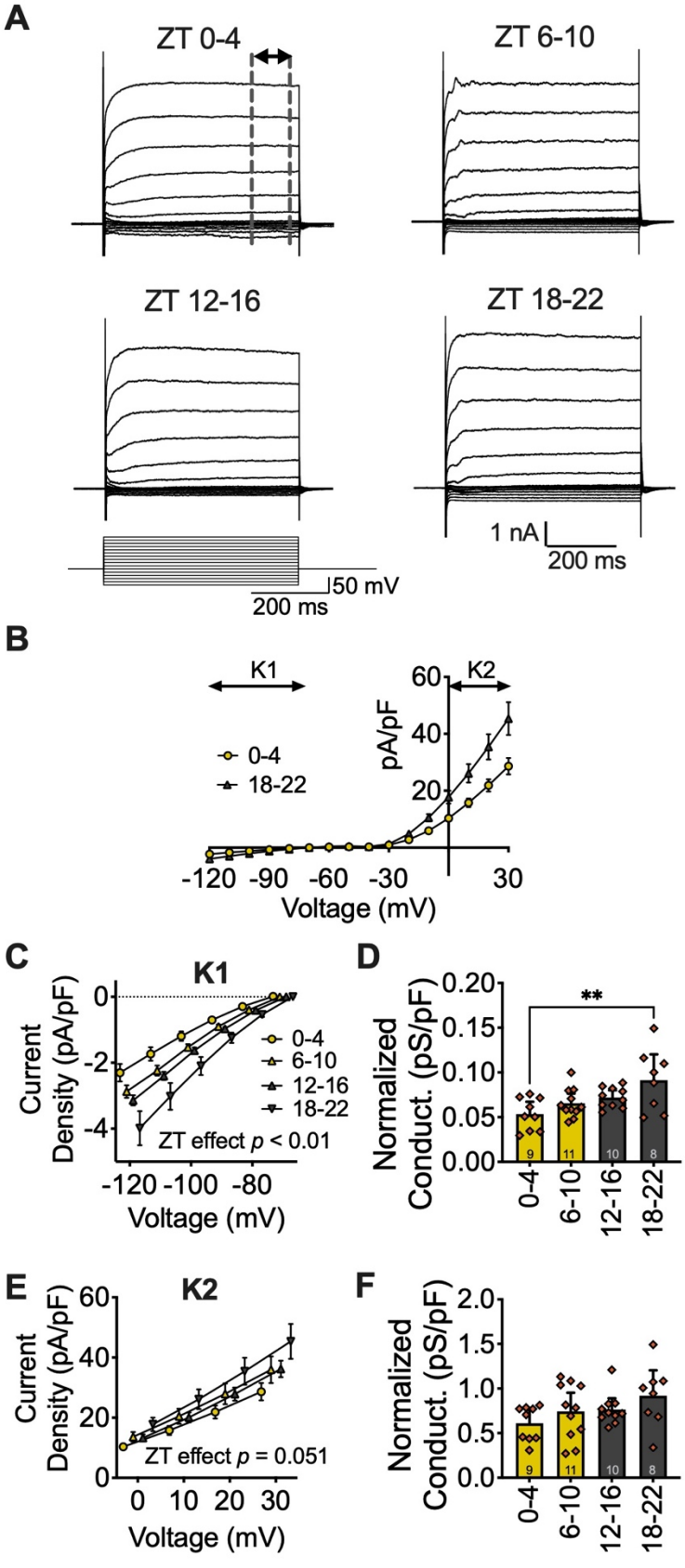

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Figure 4. *Time-of-day impact on membrane conductance and currents in female mice.* **(A)** Current-voltage trace (*top; dashed line represents steady state current averaged for analysis*) after voltage-step protocol (*bottom*). **(B)** Averaged I-V voltage-step relationship from -120mV to 30mV for ZT0-4 and 18-22 (*normalized to cell capacitance*) with a K^+ internal solution. **(C, E)** Current density and **(D, F)** conductance of K1 and K2 currents (*respectively*) at ZT0-4, 6-10, 12-16, and 18-22 in female mice. K1 and K2 represent hyperpolarized and depolarized currents (*respectively*). Error bars represent ± 95%CI. Two-way **(*C, E*)** or One-way **(*D, F*)** ANOVA with Tukey post-hoc analysis for ZT bin. $^{**}p < 0.01$. N-values for number of cells inset on bars, exact p-values and analysis in **Figure 4 – source data 1**.

Time-of-day impact on current voltage relationship in plPFC pyramidal neurons of male mice

Given that synaptic inputs are rhythmic in male and female mice, but only male mice display daily rhythms in RMP, we hypothesized that changes in postsynaptic ionic currents, as measured by the I-V relationship, may play a role in setting the functional tone of plPFC pyramidal neurons in male mice. As in **Figure 4**, we analyzed the I-V relationship by running a voltage-step protocol (**Figure 5A, B**). In male mice the I-V relationship demonstrated a clear inward rectifying current at lower holding voltages and a delayed rectifying current at depolarizing voltages, resulting in a larger current density early in the dark period (ZT12-16) in both the hyperpolarized (**Figure 5A-C**) and depolarized state (**Figure 5A, B, D**). This effect translated into higher normalized cell conductance late in the light period and early in the dark period (**Figure 5I, J)**.

Since ionic conductance was highest at ZT12-16, the same ZT bin that RMP was most hyperpolarized, we predicted that this increase involved ion channel activity that results in a net negative current. Further, in the hyperpolarized state, the current density at each ZT began to converge near our calculated reversal potential for K^+. To determine if daily changes in current density and normalized cell conductance was dependent on K^+ channel activity, we utilized a K^+ free Cs^+-based internal recording solution to block outward K^+ currents. This preparation completely abolished the time-of-day effect on current density at lower holding voltages, but not in the depolarized state (**Figure 5E-**

H). Further, the time-of-day effect on normalized cell conductance was blocked when calculated at the presented voltages with a main effect of Cs^+ on overall conductance in the depolarized state (**Figure 5I,J**). Of particular note, blockade of outward K^+ currents via internal Cs^+ appeared to have little effect at ZT0-4, suggesting minimal K^+ channel activity at this ZT bin (**Figure 5C,G**). Together, these data demonstrate that ion channel activity is time-of-day dependent in plPFC pyramidal neurons and K^+ channels contribute to daily rhythms in their cellular conductance.

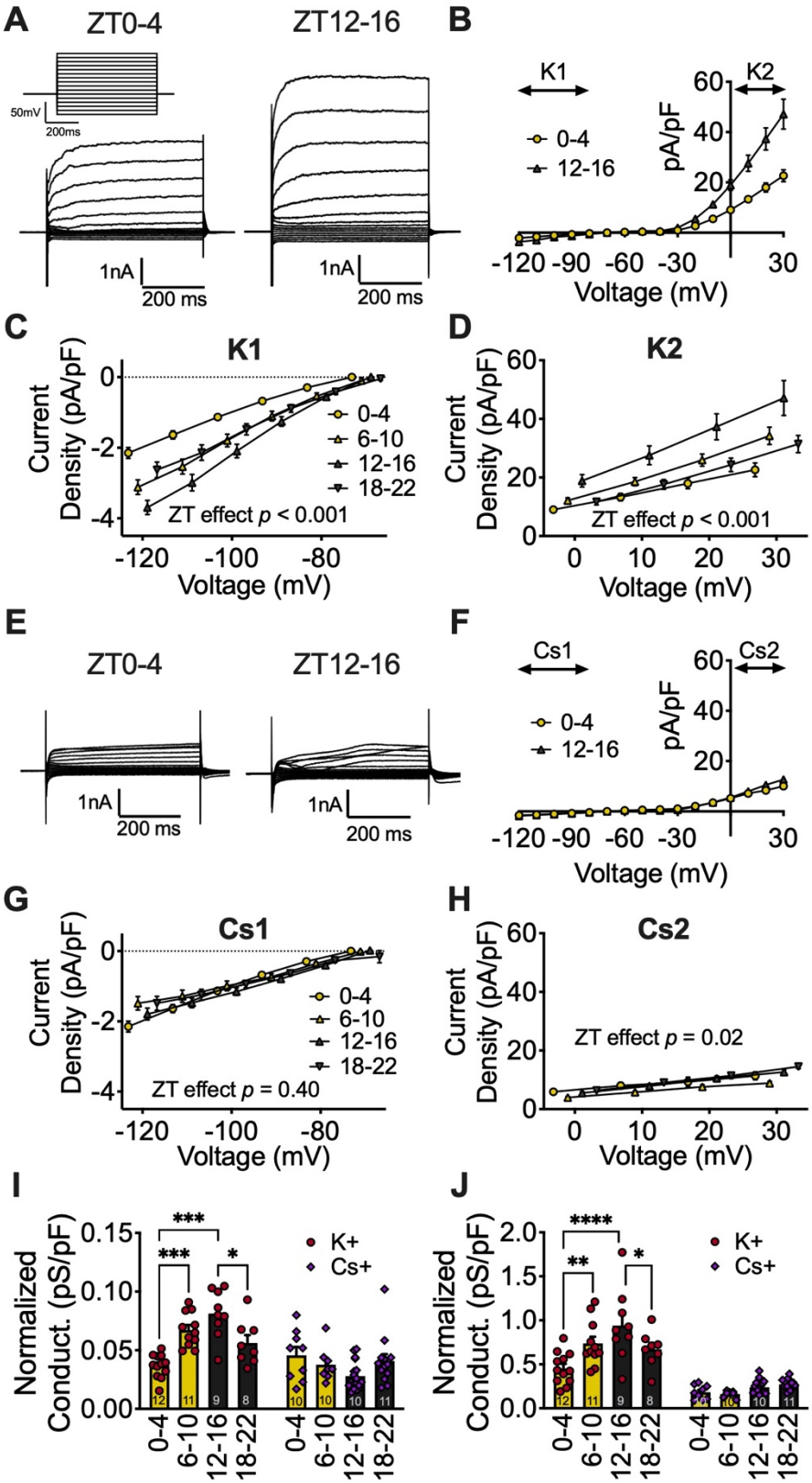

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Figure 5. *Membrane conductances are mediated by K+ channels in males*

(A) Voltage-step protocol (*top left*) and representative voltage-step traces of I-V relationship at ZT 0-4 (*left*) and 12-16 (*right*) in male mice. **(B)** Averaged I-V relationship for ZT0-4 and 12-16 (*normalized to cell capacitance*) with a K^+ internal solution. **(C)** Current density of K1 and **(D)** K2 I-V relationships at ZT0-4, 6-10, 12-16, and 18-22. **(E)** Representative voltage-step traces of **(F)** I-V relationship at ZT 0-4 (*left*) and 12-16 (*right*) with a Cs+ internal solution. **(G)** Current density of Cs1 and **(H)** Cs2 I-V relationships at each ZT bin. **(I)** Comparison each ZT bin for K1 and Cs1, and **(J)** K2 and Cs2 normalized cell conductance. Two-way ANOVA for main effects and interaction, with a within group Tukey post-hoc analysis for ZT bin, voltage, and internal solution. Error bars represent ± 95%CI and N-values for recorded cells are inset in bars. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Exact p-values, mouse N-values, and analysis in **Figure 5 – source data 1**.

Time-of-day alters excitability of plPFC pyramidal neurons

To understand the functional implications of daily rhythms in RMP and postsynaptic ion channel function for information throughput, we tested how time-of-day impacts action potential dynamics. We utilized a 10 pA current injection protocol to evoke action potentials at ZT0-4, 6-10, 12-16, and 18-22 and observed a main effect of time for membrane potential threshold of action potential firing, with post-hoc analysis revealing an increased threshold for firing late in the dark period (ZT18-22) when compared to ZT6-10 in male mice (**Figure 6A-C**). Consistent with the null effect of time on RMP in female mice, there was no effect of time on action potential threshold (**Figure 6C**). Further, although there was no effect on amount of current needed to elicit an action potential (rheobase), once rheobase was reached, subsequent current injections evoked action potential firing at a lower frequency during the dark period (**Figure 6D, E**). Although we did not observe a time-of-day effect on action potential amplitude or half-width in male mice (**Figure 6F-G**), decay tau was reduced at ZT18-22, a component of action potential firing that is modulated to a large extent by K^+ channels (**Figure 6E**). These data suggest that plPFC pyramidal neurons are not only more hyperpolarized during the light period, but are

functionally more difficult activate, requiring larger depolarizations to elicit action potentials and relay information downstream.

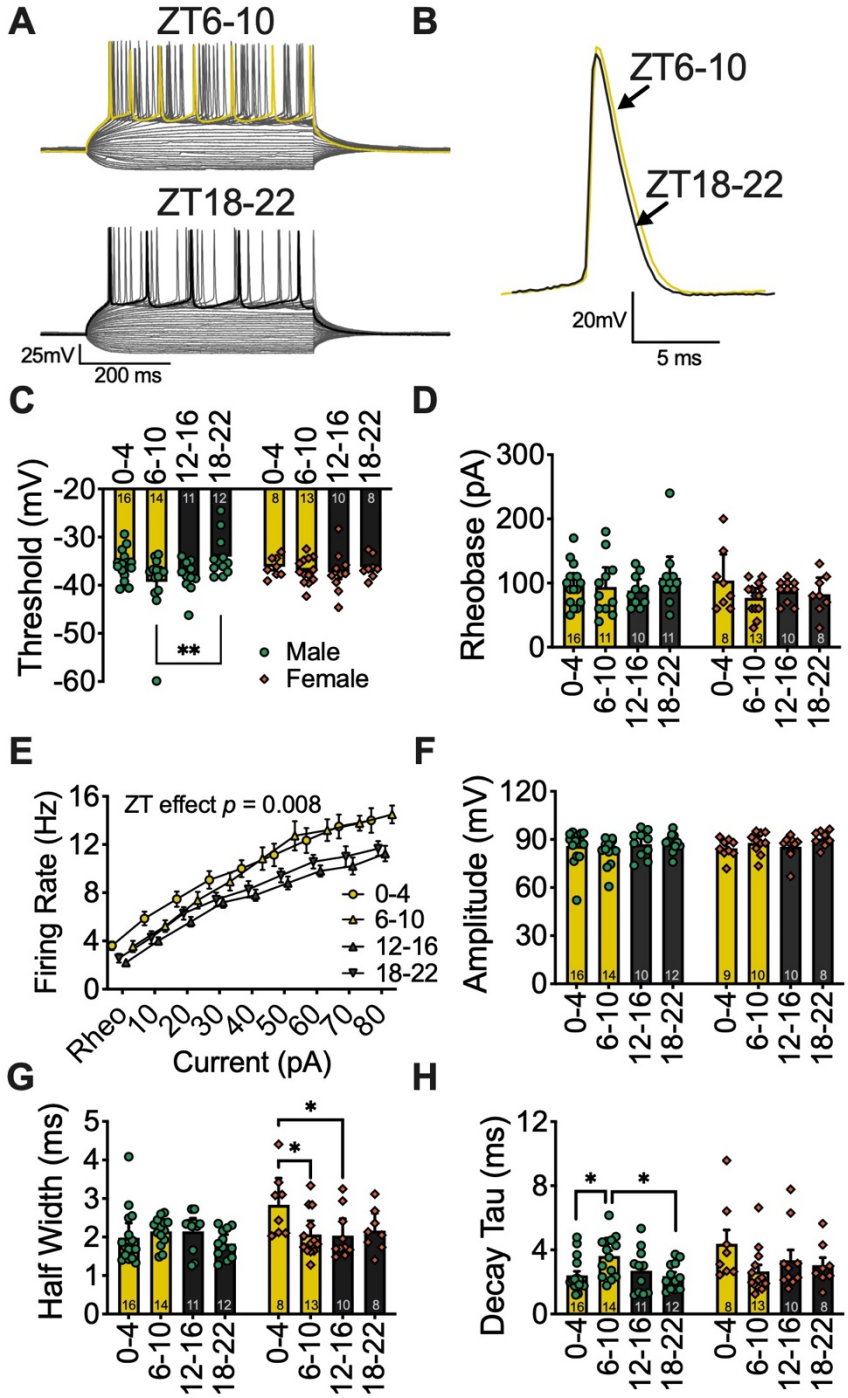

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Figure 6. *Time-of-day differences in action potential dynamics.* **(A)** Representative trace of current step recording with maximal AP firing highlighted at ZT6-10 (*top; yellow*) and 18-22 (*bottom; black*) and **(B)** individual evoked APs in male mice. **(C)** Mean AP threshold **(D)** Rheobase, **(E)** evoked firing rate (from rheobase; male mice), **(F)** amplitude, **(G)** half width, and **(H)** decay tau at each ZT bin in male and female mice. Error bars represent ± 95%CI. Two-way ANOVA for main effects and interaction, with a within group Tukey post-hoc analysis for ZT bin and/or current injection. *$p < 0.05$, **$p < 0.01$. Number of cells included in for each bar. Exact p-values, mouse N-values, and analysis in **Figure 6 – source data 1**.

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Discussion

The suprachiasmatic nucleus (SCN) is the central circadian clock in mammals and regulates daily changes in gene expression and activity throughout the brain (M. H. Hastings, Reddy, and Maywood 2003). However, it has become clear that numerous brain regions also display endogenous daily rhythms. A major hypothesis in the field is that these extra-SCN clocks and the SCN operate synergistically to drive daily rhythms in nearly all components of physiology and behavior, which is why their disruption has numerous physiological and psychological consequences, including exacerbated metabolic and mood disorders (Otsuka et al. 2020; McCarthy and Welsh 2012; Morris et al. 2015; Bechtold, Gibbs, and Loudon 2010; Karatsoreos 2012). While there have been several well executed studies that demonstrate the importance of circadian rhythms on neurophysiological function in the hippocampus and brainstem, none have included the PFC, and all have largely focused on extracellular field recordings (Chaudhury, Wang, and Colwell 2005; Loh et al. 2015; Paul et al. 2020; Chrobok et al. 2021; McMartin et al. 2021). Thus, previous studies have not shed light on the fundamental electrophysiological processes at the cellular level that are affected by time-of-day.

In this study we present four main findings. First, layer 2/3 plPFC pyramidal neurons in male mice are hyperpolarized during the early portion of the dark period when compared to the latter portion of the light period. Second, time-of-day impacts excitatory and inhibitory inputs onto plPFC pyramidal neurons, with clear sex differences in excitatory inputs. Third, we demonstrate that male mice display distinct changes in ion channel activity and action potential kinetics, with male mice having an increased action potential firing threshold and decreased decay tau during portions of the dark period. Fourth, we identify that changes in K^+ channel activity serves as a potential mechanism underlying time-of-day changes in the RMP and action potential firing rates of plPFC pyramidal neurons. By identifying the intrinsic properties and synaptic inputs of plPFC pyramidal neurons, these findings allow us to better understand the relationship between circadian rhythms, PFC circuitry and its associated behaviors.

Changes in PFC function underly numerous psychiatric disorders including bipolar, post-traumatic stress disorder (PTSD), attention deficit disorder, and deficits in learning and memory (Popoli

et al. 2012; Sotres-Bayon, Cain, and LeDoux 2006; Miller and Cohen 2001; Xu et al. 2019). There is growing evidence of links between circadian rhythms and PFC function (Otsuka et al. 2020; Woodruff et al. 2018; Hou et al. 2022; Harkness et al. 2021). Previous work from our group has demonstrated that extracellular lactate (a functional output of neural metabolism) shows circadian rhythms in the medial (m) PFC, and that environmental circadian disruption alters the morphology of medial mPFC neurons and affects PFC mediated behaviors (Wallace et al. 2020; Karatsoreos et al. 2011). However, the studies presented here are the first to explore whether cell autonomous activity and synaptic inputs onto PFC neurons are rhythmic.

Our finding that the resting state of plPFC neurons is more hyperpolarized during the dark period, when the animals are awake and active, suggests that these neurons are less active and require a higher degree of information input before eliciting a response and sending downstream signals to other brain regions. On the surface, it seems counter-intuitive that plPFC pyramidal neurons would be more inactive during the dark period, when these animals are active and engaging with their environment, than the light (inactive) period. A functional hypothesis for this finding is that stronger gating during the wake period serves as a necessary mechanism for selective informational throughput in response to environmental stimuli. Information filtering is paramount to having a proper behavioral output, and too low of a threshold may result in overactivation as the animal engages with its environment. For example, pharmacological studies have demonstrated that activation of the plPFC with neurotensin agonists or the sodium channel activator veratrine lead to anxiogenic behaviors, likely through increased glutamate release (Li, Chang, and Xi 2021; Petrie et al. 2004; Saitoh et al. 2014). Notably, we discovered a large increase in excitatory inputs onto these neurons during the active period. This is in line with other studies demonstrating that in layer 2/3 cortical neurons, excitatory inputs are increased during spontaneous wakefulness and sleep deprivation occurring during the light (inactive) period (Liu et al. 2010). While it seems contradictory that these neurons simultaneously receive more excitatory inputs and become more hyperpolarized, it is aligned with the proposal that these neurons require stronger gating mechanisms during the active period, as more information is being sent to these

283 neurons and it is critical that these incoming stimuli are somewhat filtered so only the strongest signals
284 are relayed further downstream.

285 Neurophysiological sex differences in the PFC, and their respective behavioral outputs, are well
286 documented and partly attributed to differences in synaptic signaling (Andrade et al. 2012; de Velasco
287 et al. 2015). While exploring the effects of time-of-day on these fundamental properties of PFC cells, we
288 fully embraced inclusion of both males and females, given the significant work demonstrating that
289 inclusion of both sexes (particularly inclusion of females) can reveal important new concepts and
290 understanding about brain function (Shansky and Murphy 2021). While not designed explicitly as a sex-
291 differences study, our results demonstrate that female mice had less excitatory inputs than males, yet
292 these inputs resulted in much larger postsynaptic currents, likely due to sex differences in glutamate
293 receptor expression and basal release (Perry et al. 2021). There are also sex differences in response to
294 environmental and pharmacological stressors, which are due in part to circulating sex hormones (Yuen,
295 Wei, and Yan 2016). For example, when compared to male rats, females in proestrus display a lower
296 threshold for impaired working memory after PFC injections of benzodiazepine inverse agonists that
297 activate the stress system, but this effect does not persist during estrus, when circulating estrogen
298 levels are lower (Shansky et al. 2004). Further, gonadal hormones underly sex differences in mPFC
299 dendritic growth, microglia activity, and astrocyte morphology in response to stress (Bollinger et al.
300 2019). It should be noted that there is a report that basal PFC glutamate release is higher in females,
301 which seems opposite to our findings, but is likely due to experimental differences, as these studies
302 differ in species, PFC layers, electrophysiological solutions, and time-of-day (Pena-Bravo et al. 2019).
303 We speculate that if the underlying mechanisms that mediate information throughput and plasticity are
304 fundamentally different in males and females, and the basal tone of excitatory inputs is relatively low in
305 females, then time-of-day changes in information filtering may not be as crucial to optimal plPFC
306 function in female mice.

307 GABAergic interneurons are highly involved in PFC function and relay information between
308 multiple regions of the PFC (Saffari et al. 2016; Hu, Gan, and Jonas 2014; Anderson et al. 2021). In the
309 hippocampus, GABAergic inputs onto CA1 pyramidal neurons regulate action potential firing frequency

in response to current injections, with a higher inhibitory tone during the light cycle (Fusilier et al. 2021; Albers et al. 2017). Significantly, we found that in the plPFC there was no effect of sex or time-of-day on spontaneous inhibitory inputs. This suggests that presynaptic inhibitory and excitatory inputs are not the primary regulator of resting state or information throughput in layer 2/3 plPFC pyramidal neurons. Instead, this supports the notion that basal inhibitory tone remains relatively constant throughout the 24h day and, in male mice, a postsynaptic cell endogenous mechanism is responsible for maintaining proper information filtering when these neurons are challenged by the large increase of excitatory signals that come in during the active period.

Although the frequency and amplitude of spontaneous inhibitory inputs did not change when probed by individual ZT bins, further investigation revealed that when grouped by the light/dark cycle, the strength (amplitude) of inhibitory inputs was stronger during the dark (active) period, specifically in male mice. GABA receptors interact with postsynaptic ion channels and there are sex differences in the expression of GABA receptor subunits, as well as how they interact with ion channels. For example, the steroid hormone progesterone increases the expression of the $GABA_A$ receptor subunit α1 in the PFC of rodents, and in humans, alcoholism is suggested to result in larger decreases of cortical GABA(A) receptor subunits in females than males (Andrade et al. 2012; Janeczek et al. 2020). After investigating GABAergic signaling localized at the synapse and isolated from upstream activity, we confirmed that inhibitory postsynaptic currents are stronger early in the dark period in male mice. Interestingly, female mice displayed a large increase in inhibitory inputs and strength late in the light period, a finding that requires future studies to fully understand its functional implications.

In total, our findings point toward a postsynaptic mechanism underlying daily changes in the physiology of layer 2/3 plPFC pyramidal neurons and previous work has shown that sleep deprivation can alter the intrinsic excitability of layer 5 PFC pyramidal neurons (Yan et al. 2011). This prompted us to explore how time-of-day impacts intrinsic postsynaptic properties such as ionic currents and overall conductance. At hyperpolarized voltage steps below the reversal potential of K^+, time-of-day did have a modest impact the conductance and current density of plPFC pyramidal neurons in female mice between the transition from the dark to light period. However, at depolarizing voltages greater than the

mean RMP and action potential threshold there was no time-of-day effect. This further supports the notion that while physiological daily rhythms do exist in plPFC pyramidal neurons of female mice, they have little impact on overall resting state and information throughput in response to electrical activity and synaptic inputs. In contrast, there was a large effect on the current density and conductance of pyramidal neurons in male mice. Specifically, when these neurons were hyperpolarized below the equilibrium potential for K^+, we discovered that current density increased throughout the light period, peaking between the late period and early dark period. This effect translated into an overall increase in conductance, and given that conductance was highest around the beginning of the active period, when these neurons are most hyperpolarized, we presumed that this was due to an increased number of open K^+ channels and the outflow of K^+ cations (outward current). Consistent with this prediction, when we replaced K^+ with Cs^+ in our internal recording solution (to block K^+ channel mediated outward currents), the time-of-day effect on current density and conductance was completely abolished at voltages near or below the K^+ equilibrium potential. Although internal Cs^+ was not sufficient to block the time-of-day effect on current density at depolarized voltage greater than the K^+ equilibrium potential, it greatly reduced overall current density and conductance. Moreover, previous studies demonstrate that internal Cs^+ is not sufficient to block the inward K^+ currents expected at voltages above the K^+ equilibrium potential (Adelman and Senft 1966).

To understand the functional relevance of these postsynaptic changes in ion channel function and resting state, it was necessary to determine how time-of-day impacts action potential dynamics as action potential firing is a functional measure for information throughput. Notably, this measure changes with time-of-day in the hippocampus, and in response to sleep deprivation in the PFC (Fusilier et al. 2021; Yan et al. 2011). Action potentials are dependent on voltage-gated ion channels, and changes in K^+ channel activity can alter action potential firing threshold and kinetics. Consistent with our interpretation that, in male mice, there is a stronger gating mechanism to filter incoming signals during the active period, we discovered that the threshold for action potential firing was increased during the active period. These data suggest that layer 2/3 plPFC pyramidal neurons are not only more hyperpolarized during the light period, but are functionally more difficult to activate, requiring much

larger depolarizations to elicit action potentials and relay information downstream. Though somewhat speculative, this could affect a wide range of behaviors, including emotionality, a notion supported by work demonstrating that pharmacological activation of plPFC neurons induces anxiogenic activity in mice, and acute stress enhances glutamatergic transmission in the PFC (Yuen et al. 2009; Li, Chang, and Xi 2021; Saitoh et al. 2014). Further, alcohol is a common drug of abuse in those suffering from PFC associated affective pathologies, and in vivo electrophysiology studies show that alcohol preferring rats have higher baseline neural firing in the PFC (Linsenbardt and Lapish 2015).

Combined, we believe this work demonstrates the importance of understanding how daily rhythms impact neural function, which is necessary to fully grasp the relationships between brain and behavior. It is critical to recognize that the mPFC is heterogeneous at the anatomical and physiological levels, with consequences for behavior (Moorman et al. 2015). Our work suggests that even when looking at the fundamental properties of cellular function in the mPFC, perhaps we need to add heterogeneity at the temporal level as well. To fully appreciate the relationship between brain, behavior, and daily rhythms, future studies are required to determine how these rhythms impact communication with extra- (such as the hippocampus and amygdala) and intra- (such as the infralimbic) PFC regions. Additionally, future studies are necessary to determine exactly which ion channels are mediating daily changes in PFC function and how environmental factors that alter whole-animal physiology and behavior, such as circadian disruption, may impact these circuits. Given the impact of time-of-day on neuronal function in the PFC, the work presented here also has significant implications for incorporating time-of-day into the application of pharmacological and behavioral interventions for mental health disorders, and opens the door to similar questions in brain regions outside the PFC.

Methods

Animals

All animal procedures and experiments were approved by the University of Massachusetts Amherst Institutional Care and Use Committee in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Male and female wild-type mice (Charles River, Wilmington, MA, USA) on a C57BL/6J background were used for these studies. All mice were group-housed in light boxes at 25°C, under a 12/12-hr light/dark (LD) cycle, with food and water available *ad libitum*. Light box LD cycles were offset so that experiments from each ZT bin occurred at the same time each day. Mice ages 10-16 weeks were used for these studies. For electrophysiology studies mice were anesthetized in a chamber with isoflurane before euthanasia by decapitation.

Brain slice electrophysiology

Two mice were simultaneously euthanized 1-hr prior to their ZT bin (i.e., mice were euthanized at ZT23 for recording bin ZT0-4). After euthanasia, brains were immediately removed and the forebrain was blocked while bathing in a 0-4°C oxygenated N-methyl-D-glucamine (NMDG) - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) cutting solution composed of (mM): 92 NMDG, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 3 sodium pyruvate, 2 thiourea, 20 HEPES, 10 $MgSO_4$, 0.5 $CaCl_2$, 25 glucose, 20 sucrose. Cutting solution was brought to pH 7.4 with ~17mL of 5M HCl (Ting et al. 2018). The forebrains were mounted adjacent to each other and sectioned simultaneously on a vibratome (VT1200S, Leica Biosciences, Buffalo Grove, IL, USA) with a sapphire knife (Delaware Diamond Knives, Wilmington, DE, USA) yielding roughly three slices containing the PFC from each (250-µm) per mouse. Slices were transferred and allowed to recover for 30-45 min in room temperature recording artificial cerebrospinal fluid (aCSF) solution composed of (mM): 124 NaCl, 3.7 KCl, 2.6 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 2 $MgSO_4$, 10 glucose. aCSF had a final pH of 7.3-7.4, osmolarity of 307-310 mOsmos, and was continuously bubbled using 95% 0_2/5% $C0_2$. For recordings, brain slices were transferred to a perfusion chamber containing aCSF maintained at 34-37°C with a flow rate of 1mL/min.

Neurons were visualized using an upright microscope (Zeiss Axoskop 2, Oberkochen, Germany). Recording electrodes were back-filled with experiment-specific internal solutions as follows (mM): Current-clamp and spontaneous (s) excitatory postsynaptic currents (EPSCs); 125 K-gluconate, 10 KCl, 10 NaCl, 5 HEPES, 10 EGTA, 1 $MgCl_2$, 3 NaATP and 0.25 NaGTP (liquid-junction potential (LJP) = ~14.5 mV). Voltage-clamp spontaneous inhibitory postsynaptic currents (sIPSCs); 140 CsCl, 5 $MgCl_2$, 1 EGTA, 10 HEPES, 3 NaATP, and 0.25 NaGTP (LJP = ~4.2 mV). All internal solutions were brought to pH 7.3 using KOH (current-clamp and EPSC) or CsOH (IPSC) at 301-304 mOsm. EPSCs were recorded in the presence of the GABA receptor antagonist bicuculline (30 μM). sIPSCs were recorded in the presence the competitive α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM), the selective N-Methyl-d-aspartate (NMDA) receptor antagonist (2R)-amino-5-phosphonovaleric acid (APV; 50 μM), and the glycine antagonist strychnine (2 μM). Miniature (m) IPSCs had the addition of the voltage-gated sodium channel (VGSC) blocker tetrodotoxin (TTX; 1 μM). Patch electrodes with a resistance of 3-5MΩ were guided to neurons with an MPC-200-ROE controller and MP285 mechanical manipulator (Sutter Instruments, Novato, CA, USA). Patch-clamp recordings were collected through a UPC-10 USB dual digital amplifier and Patchmaster NEXT recording software (HEKA Elektronik GmbH, Reutlingen, Germany). All voltage-clamp recordings were obtained at V_H= -70mV. Current clamp voltage-step protocols were performed from the cell endogenous resting membrane potential, and used 500ms 10pA steps from -100pA to +190pA. Voltage clamp current-step protocols were performed from V_H= -70mV, and used 10mV steps from -120mV to +30mV. All compounds were obtained from Tocris Cookson, Cayman Chemical, and Sigma Aldrich.

Individual recording locations were plotted (with neurons outside of the target area excluded from analysis) as well as to qualitatively confirm an equal distribution of recording sites between Zeitgeber (ZT) bins 0-4, 6-10, 12-16, and 18-22 (**Figure 1 – figure supplement 1A-D**). A small percentage (~20%) of all recorded neurons had unique characteristics in resting membrane properties, spontaneous excitatory postsynaptic currents (sEPSCs), and action potential dynamics that were independent of time-of-day (hereafter Type II neurons; **Figure 1 - figure supplement 2A-I**). Most

notably, compared to Type I (most abundant) neurons, Type II neurons (less abundant) displayed a much higher action potential velocity and hyperpolarizing decay current (**Figure 1 - figure supplement 2A, B**). They also had a more depolarized resting membrane potential (RMP) and decreased action potential firing threshold (**Figure 1 - figure supplement 2E, F**). Due to these clear qualitative and quantitative differences independent of ZT bin, and that they represented a small proportion of recorded neurons, we excluded the far less abundant Type II neurons from analysis in our following experiments.

Statistical Analysis

For sEPSCs, only neurons with holding currents not exceeding 100pA at V_H= -70mV for the 10-min control period (input resistance > 70 MΩ) were studied further. Neurons were not considered for further analysis if series resistance exceeded 50MΩ or drifted >10% during baseline. Rheobase was calculated as the first current step to elicit an action potential and action potential dynamics (threshold, decay tau, and half-width) were obtained from the first evoked action potential to avoid variance in ion channel function due to repeated action potential firing. G*Power 3.0 software (Franz Faul, Uni Kiel, Germany) was used to conduct our power analysis, for a *p* value of <0.05 with 90% power. Adequate sample sizes were based upon expected effect sizes from similar experiments. Raw data files were analyzed in the Patchmaster NEXT software or converted using ABF Utility (Synaptosoft) for analysis in Clampfit (Molecular Devices, San Jose, CA, USA). N-values for analysis and presented in figures represent individual cells. To control for biological variability between groups N = 4-8 mice per group (see figure source data). Statistical comparison of effects between each time-period was made using a full model two-way ANOVA (column, row, and interaction effects) for comparison of the current-voltage relationships and comparing the interaction and main effect of time and sex or internal solution. For all experiments, error bars are presented as mean ± 95% confidence interval (CI). Statistics were calculated using Prism 9 (Graphpad Software, San Diego, CA, USA).

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Competing Interests

BLR, JW and INK declare no competing financial interests.

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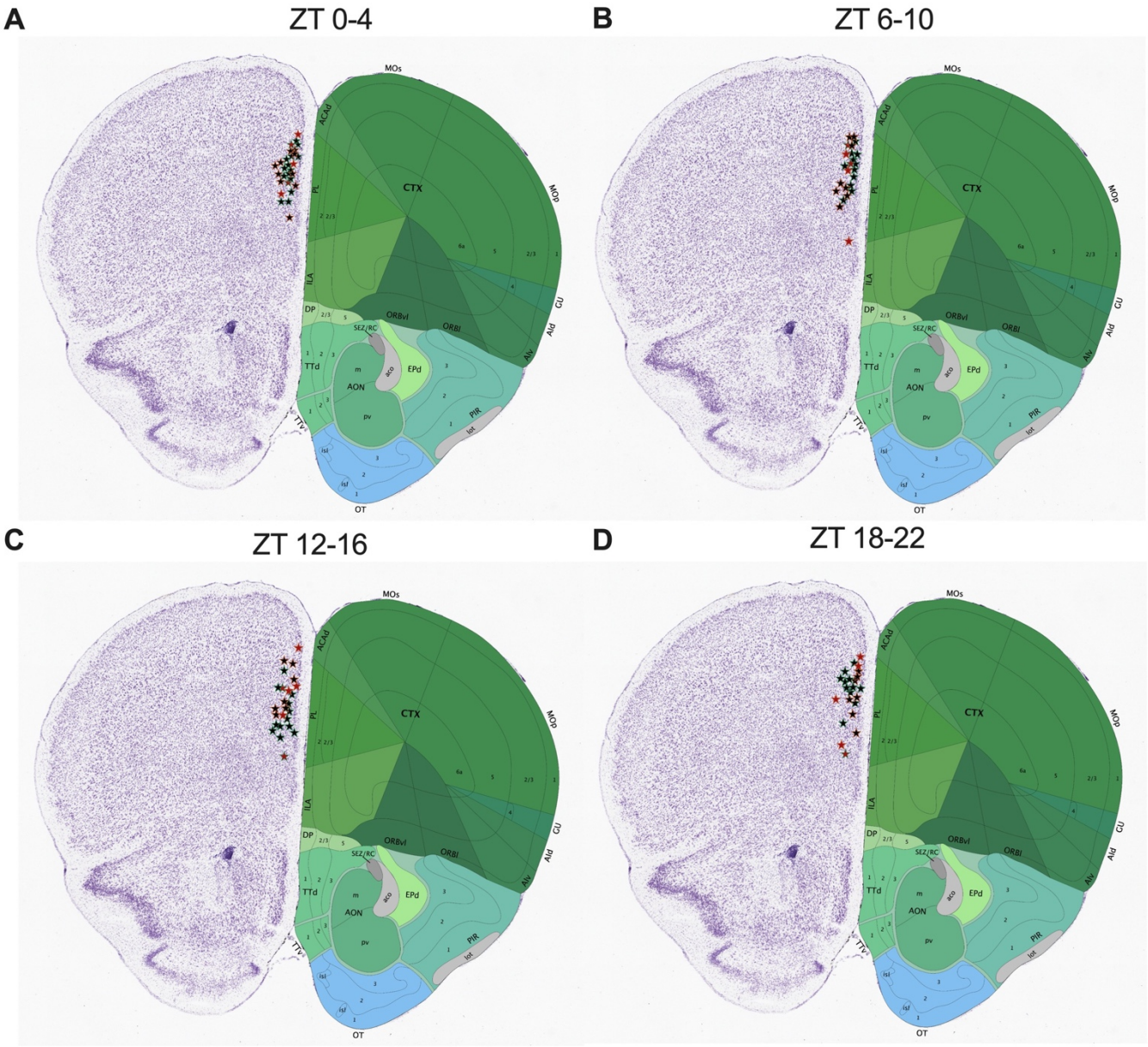

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Figure 1 – figure supplement 1. *Recording map for layer 2/3 plPFC pyramidal neurons.* Coronal sections of forebrain showing individual recording sites from majority of neurons that were imaged at **(A)** ZT0-4, **(B)** 6-10, **(C)** 12-16 and **(D)** 18-22 for basal membrane property, sEPSC, and evoked action potential experiments in male (*bluish green outline*) and female (*vermillian outline*) mice. Stars filled with black represent 'Type I' neurons included for analysis and red stars represent Type II/III neurons excluded from analysis.

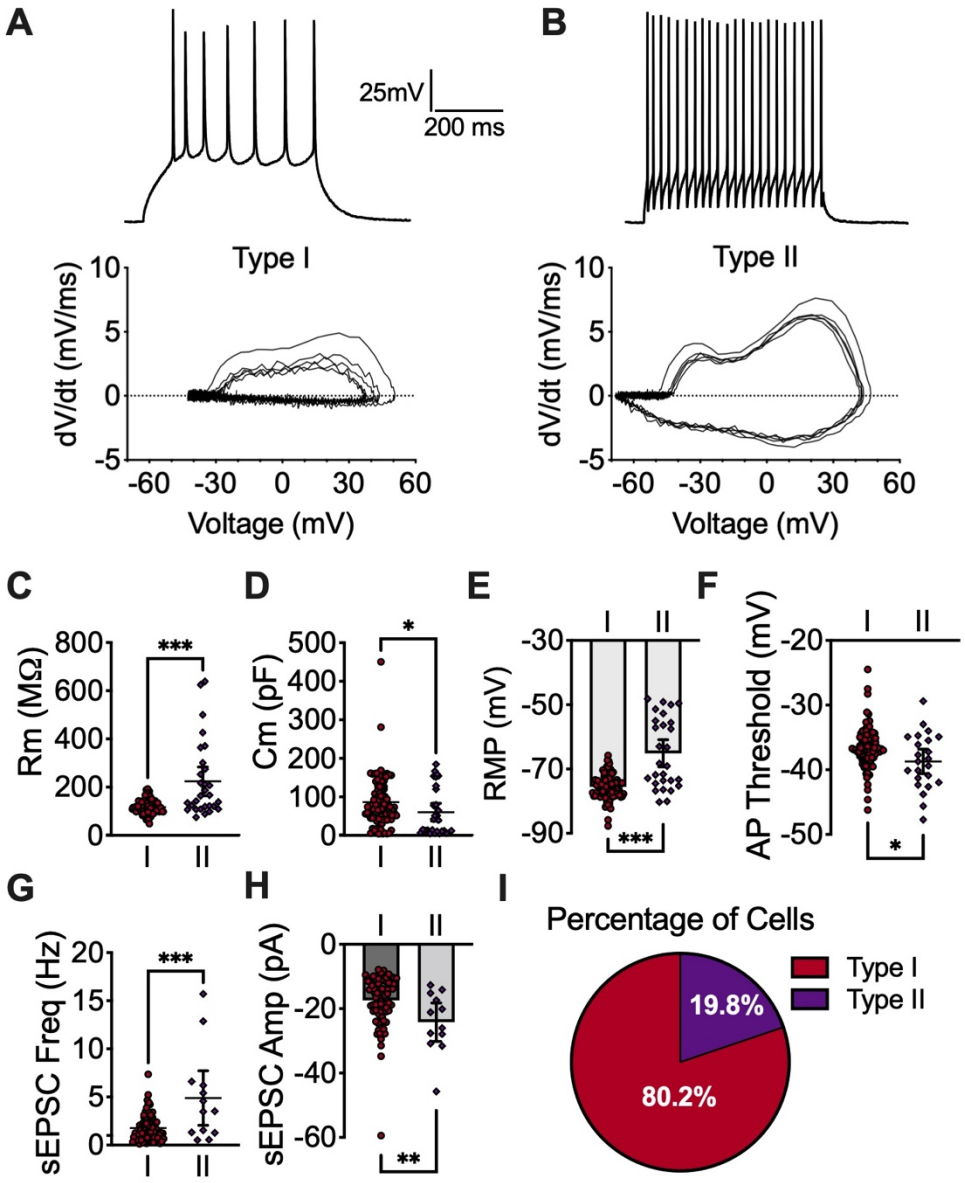

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Figure 1 – figure supplement 2. *Categories and distinct physiological characteristics of plPFC neurons.* **(A)** Representative evoked action potential traces and phase plot diagram of first five action potentials (*bottom*) illustrating differences in velocity, trajectory, and amplitude in Type I and **(B)** Type II neurons. **(C)** Comparison of membrane resistance (Rm), **(D)** membrane capacitance (Cm), **(E)** resting membrane potential (RMP), **(F)** action potential (AP) threshold, **(G)** sEPSC frequency (Freq), and **(H)** sEPSC amplitude (Amp). **(I)** Percentage of recorded cells displaying Type I or Type II characteristics (combined among all ZT bins; calculated by *n* values from AP threshold). Error bars represent ± 95%CI. Unpaired student t-test, * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$. Exact p-values and analysis in **Figure 1 – supplemental source data 1**.