Neural correlates and determinants of approach-avoidance conflict in the prelimbic prefrontal cortex

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ABSTRACT

The recollection of environmental cues associated with threat or reward allows animals to select the most appropriate behavioral responses. Neurons in the prelimbic cortex (PL) respond to both threat- and reward-associated cues. However, it remains unknown whether PL regulates threat-avoidance vs. reward-approaching responses when an animals’ decision depends on previously associated memories. Using a conflict model in which rats retrieve memories of shock- and food-paired cues, we observed two distinct phenotypes during conflict: i) rats that continued to press a lever for food (Pressers); and ii) rats that exhibited a complete suppression in food seeking (Non-Pressers). Single-unit recordings revealed that increased risk-taking behavior in Pressers is associated with persistent food-cue responses in PL, and reduced spontaneous activity in PL glutamatergic (PL\textsuperscript{GLUT}) neurons during conflict. Activating PL\textsuperscript{GLUT} neurons in Pressers attenuated food-seeking responses in a neutral context, whereas inhibiting PL\textsuperscript{GLUT} neurons in Non-Pressers reduced defensive responses and increased food approaching during conflict. Our results establish a causal role for PL\textsuperscript{GLUT} neurons in mediating individual variability in memory-based risky decision making by regulating threat-avoidance vs. reward-approach behaviors.
INTRODUCTION

The brain’s ability to identify and discriminate cues associated with threat or reward allows organisms to respond appropriately to changes in the environment (Schultz, 2015; Hu, 2016). Animals respond to threatening cues with a series of defensive behaviors including avoidance responses that decrease their chances of being exposed to aversive outcomes (McNaughton and Corr, 2014; Krypotos et al., 2015; Cain, 2019). In contrast, reward cues have attractive and motivational properties that elicit approach behavior (Robinson and Flagel, 2009; Morales and Berridge, 2020). When animals are exposed to threat and reward cues simultaneously, an approach-avoidance conflict emerges, and decision-making processes are recruited to resolve the situation (Kirlic et al., 2017; Barker et al., 2019). While many studies have investigated the neural mechanisms that control threat-avoidance and reward-approach independently of each other, it is unclear how the brain uses previously learned information to regulate the opposing behavioral drives of avoiding threats and seeking rewards during a conflict situation.

Neurons in the prelimbic (PL) subregion of the medial prefrontal cortex (mPFC) change their firing rates in response to cues that predict either threat or reward (Baeg et al., 2001; Burgos-Robles et al., 2009; Burgos-Robles et al., 2013; Moorman and Aston-Jones, 2015; Dejean et al., 2016; Otis et al., 2017). Accordingly, activity in PL neurons is necessary for the retrieval of both food- and threat-associated memories (Sierra-Mercado et al., 2011; Courtin et al., 2014; Sangha et al., 2014; Do-Monte et al., 2015; Otis et al., 2017). PL neurons are reciprocally connected with the basolateral nucleus of the amygdala (BLA) (McDonald, 1991; Vertes, 2004), a region implicated in the
detection of threats or rewards (Amir et al., 2015; Namburi et al., 2015; Beyeler et al., 2016; Zhang et al., 2020). During a risky foraging task in rats, dynamic modifications in the activity of PL and BLA neurons correlate with the detection of imminent threats and the defensive readiness for action (Kim et al., 2018; Kyriazi et al., 2020). In addition, during a modified Pavlovian cue discrimination task involving footshocks as punishment, increased activity in the BLA-PL pathway is sufficient and necessary for the expression of freezing responses (Burgos-Robles et al., 2017), a passive form of defensive behavior. Conversely, inhibitory signaling in PL neurons correlates with threat avoidance (Diehl et al., 2018), an active form of defensive behavior. While these studies suggest a potential role of PL during motivational conflict involving states of certainty (i.e., imminent threats), it is unknown whether changes in PL activity underlie the behavioral variability in approach-avoidance responses under states of uncertainty, when decision depends entirely on the retrieval of associated memories. It is also unclear whether PL activity is necessary to coordinate appropriate behavioral responses during conflict, and if so, which sub-types of PL neurons govern the competing demands of approaching rewards vs. avoiding potential threats.

To address these questions, we designed an approach-avoidance conflict test that assess the ability of rats to remember cues previously associated with either food or footshocks to make a behavioral decision. Using a combination of optogenetics and single-unit recordings, we investigated rats’ individual variability in reward seeking and defensive responses during the conflict test and correlated their behaviors (e.g. freezing, avoidance, risk-assessment) with the firing rate of photoidentified glutamatergic and GABAergic neurons in PL. We then examined the role of PL neurons
in risky decision-making by optogenetically manipulating PL activity with high temporal
resolution and cell-type specificity during the conflict test.

RESULTS

Rats show individual variability in reward-seeking and defensive responses
during the approach-avoidance conflict test.

To investigate the motivational conflict between approaching rewards and avoiding
potential threats, we established a behavioral model in which rats need to balance food
seeking with conditioned defensive responses based on their memories of previously
acquired cues. Food-restricted rats (18 g of chow per day) were initially placed in an
operant box and trained to press a lever for sucrose in the presence of audiovisual cues
that signaled the availability of food. Each lever press during the audiovisual cue
presentation resulted in the delivery of a sucrose pellet into a nearby dish (see Methods
for details). When rats reached 50% of discrimination during cued food-seeking, they
began lever-pressing for sucrose preferentially during the audiovisual cues
(Supplementary Fig. 1A-B). During the habituation day, rats were placed in an odor
arena and familiarized with the food cues and the neutral odor amyl acetate (see
Methods for details). Next, to pair the odor cue with an aversive stimulus, rats were
exposed to an olfactory threat conditioning training (Day 1). Animals were placed in an
operant box (conditioning box; Fig. 1A left) previously connected to an olfactometer
and habituated to one odor presentation (amyl acetate, 30 s) without footshock,
followed by five odor presentations of the same odor that co-terminated with an
electrical footshock (0.7 mA, 1 s duration, 270-390 s inter-trial intervals, Fig. 1A far-left). Food cues (30 s duration) were presented during the inter-trial intervals to assess lever press suppression. Rats showed robust defensive responses during the threat conditioning training, as evidenced by an increase in freezing during the conditioned odor presentation (Fig. 1B), a decrease in lever presses (Fig. 1C), and an increase in the latency to press the lever (Fig. 1D) during the presentation of the food cues across trials. After rats have acquired the reward and threat associations, they were returned to the same odor arena in which they were previously habituated and exposed to a test session (Day 2) (Fig 1A, right). The test session consisted of three different phases: (i) a Reward Phase, in which only the audiovisual cues signaling the availability of food were presented; (ii) an Odor Phase, in which only the conditioned odor was presented, and a (iii) Conflict phase, in which both the food cues and the conditioned odor were presented simultaneously (Fig 1A, far-right).

During the reward phase, rats spent ~40% of the time in the food area and pressed the lever for food in ~95% of the food cue trials, without exhibiting significant defensive behaviors (Fig. 1E-J). Introduction of the shock-paired odor during the odor phase reduced the percentage of time rats spent in the food area to ~15%, and increased defensive behaviors characterized by heightened freezing, avoidance, and risk-assessment responses (Fig. 1E-H). These defensive behaviors were attenuated by the introduction of food cues during the conflict phase, as evidenced by a reduction in the percentage of time avoiding the threatening odor (Fig. 1F) and an increase in the percentage of time approaching the food area (Fig. 1H). This indicates that the concomitant presentation of food cues and shock-paired odor induced a behavioral
conflict in the animals. Interestingly, when we analyzed the percentage of rewarded
presses during the conflict phase (Fig. 1J), two behavioral phenotypes emerged: i) rats
that continued to press the lever for food in the presence of the threatening odor
(Pressers, Fig. 1K); and ii) rats that showed a complete suppression in lever presses in
the presence of the threatening odor (Non-Pressers, Fig. 1L). We then separated the
animals into two different groups and compared their behaviors during the entire test
session (Fig. 1K-R, Supplementary Movie 1). While Pressers and Non-Pressers
showed similar behavioral responses during the reward phase (all p values > 0.05),
Pressers showed a lower percentage of time exhibiting freezing and avoidance
responses (Fig. 1M,N), and a greater percentage of time approaching the food area
(Fig. 1P) when compared to Non-Pressers during both the odor and the conflict phases.
These individual phenotypes were not due to prior behavioral differences between the
two groups because Pressers and Non-Pressers showed similar lever pressing rates
during the cued food-seeking training, as well as the same levels of freezing, velocity
(i.e., maximum speed), and lever presses during the threat conditioning phase
(Supplementary Fig. 1A-E), indicating that increased risk-taking behavior in Pressers
was not caused by prior differences in reward-seeking motivation. Together, our results
demonstrate that our conflict model is a suitable paradigm to investigate the interactions
between reward- and threat-associated memories. Given that rats exhibit individual
differences in food seeking and defensive responses during the test session, we next
took advantage of the two observed phenotypes to examine the neuronal correlates of
risk-taking (Pressers) and risk-avoiding (Non-Pressers) behaviors in PL neurons.
PL neurons respond differently to reward cues in Pressers vs. Non-Pressers during conflict.

To investigate the role of PL neurons in regulating food-approach and threat-avoidance responses, we performed single-unit recordings across the different phases of the conflict test (Fig. 2A). We aligned the activity of PL neurons to the onset of the food cues during the reward phase and tracked the firing rates of the same cells during the conflict phase. Using the behavioral classification shown in Fig. 1J, we separated the animals into Pressers or Non-pressers and compared changes in PL activity in response to food cues during the reward and conflict phases (Fig. 2B-V). When PL activity was time-locked to the onset of the food cues during the reward phase, Pressers showed a higher number of food-cue responsive neurons than Non-Pressers (Fig. 2C-D vs. Fig. 2M-N), with the proportion of excitatory and inhibitory responses being similar between the two groups. During the conflict phase, both Pressers and Non-Pressers showed a significant reduction in the number of food-cue responsive neurons (Fig. 2C-D vs. 2M-N), suggesting that PL neurons can distinguish between reward and conflict situations. However, Pressers exhibited persistent excitatory food cue responses (Fig. 2E vs. Fig. 2O), which were higher in magnitude than in Non-Pressers (Fig. 2F vs. Fig. 2P). In addition, Pressers showed a higher magnitude of inhibitory food-cue responses during the reward phase and, in contrast to Non-Pressers, such responses were attenuated during the conflict phase (Fig. 2FI-J vs. Fig. 2S-T).

Next, we time-locked the activity of PL neurons to the onset of the food cues during the conflict phase. We observed a larger number of food-cue responsive neurons in Pressers compared to Non-Pressers (Supplementary Fig. 2A-B vs. Supplementary
Moreover, Pressers showed a higher magnitude of excitatory food cue responses during the conflict phase compared to Non-Pressers (Supplementary Fig. 2C-F vs. Supplementary Fig. 2M-P), while the magnitude of the inhibitory food cue responses in Pressers were higher during the conflict phase compared to the reward phase (Supplementary Fig. 2G-J vs. Supplementary Fig. 2Q-T).

To further explore whether changes in activity dynamics of PL neurons differ between Pressers and Non-Pressers, we compared the spontaneous firing rate of the neurons before vs. after each phase of the test session (Supplementary Fig. 3A). While Pressers showed the same proportion of excitation and inhibition across the different phases, Non-Pressers exhibited a significant increase in the proportion of excitation during the conflict phase (Supplementary Fig. 3B). This suggests that increased spontaneous activity in PL neurons during the conflict phase may be associated with the complete suppression in lever presses observed in Non-Pressers (Fig. 1J). Collectively, these results suggest that differences in the number and magnitude of excitatory food cue responses, as well as in the spontaneous activity of PL neurons, during the conflict phase may contribute to the individual differences in risky decision-making observed between the two behavioral phenotypes.

Different subsets of PL neurons signal freezing, avoidance, and risk-assessment behaviors in both Pressers and Non-pressers.

To investigate whether PL activity correlates with the expression of distinct defensive behaviors during the test session, we used a pose estimation algorithm (Deep Lab Cut, see Methods for details) to identify the onset of freezing, avoidance or risk-assessment
responses and align these time points with the activity of PL neurons. We found that a small percentage of PL neurons changed their firing rates during the onset of freezing (Fig. 3A), avoidance (Fig. 3B), or risk-assessment (Fig. 3C) behaviors in both Pressers and Non-Pressers, with a similar proportion of excitatory and inhibitory responses being observed in the two groups (Fig. 3A-I). Interestingly, most PL responsive neurons (80%) changed their activities exclusively during the onset of one of these three behaviors, with avoidance-responsive cells also responding during the onset of risk-assessment behavior (Fig. 3J-M). These results suggest that different subsets of PL neurons signal distinct behavioral outcomes during a conflict situation, with only a reduced number of PL neurons encoding the aversive salience of environmental cues independently of the behavioral defensive response expressed by the animal.

Pressers and Non-Pressers show significant differences in delta and theta oscillations in PL

Previous studies have shown that oscillations in mPFC neuronal activity at different frequency bands correlate with distinct behavioral states in both rodents and humans (Narayanan et al., 2013; Harris and Gordon, 2015). Neural oscillations in the mPFC emerge from the network of excitatory and inhibitory synaptic connections and are thought to contribute to neural communication when subjects engage in reward and threat memory tasks (Hyman et al., 2011; Likhtik and Paz, 2015; Park and Moghaddam, 2017; Widge et al., 2019). To investigate whether Pressers and Non-Pressers show significant differences in PL oscillations during conflict, we recorded local field potentials (LFPs) from PL neurons and calculated the average of power spectral density (PSD) at
different frequencies across the test session. After comparing the PSD contribution for each frequency range in Pressers and Non-Pressers, we observed that most of the signal was prevenient from the delta (0-4 Hz) and theta (4-10 Hz) bands, with a much smaller contribution coming from the alpha (10-14 Hz), beta (14-35 Hz), and gamma (>35 Hz) frequencies (Fig. 4A). We therefore focused our analyses on these two bands and found that Pressers displayed increased power in the delta band, whereas Non-Pressers exhibited increased power in the theta band during the three phases of the test session (Fig. 4B-C). Differences between Pressers and Non-Pressers were also observed in the time-frequency domain through changes in the log of PSD for delta and theta bands across the different phases (Fig. 4D-E). These results indicate that phenotypic differences in approach-avoidance conflict are associated with distinct oscillatory frequencies in PL.

In pressers, PL\textsuperscript{GLUT} neurons show reduced spontaneous activity during the conflict phase.

The rodent mPFC, including PL, is primarily composed of excitatory glutamatergic cells that correspond to 75-85% of the neurons in this area. In contrast, inhibitory GABAergic interneurons comprise 15-25% of the local neurons (Santana et al., 2004; Gabbott et al., 2005). Previous studies have shown that PL glutamatergic (PL\textsuperscript{GLUT}) neurons are necessary for the retrieval of conditioned threat responses (Do-Monte et al., 2015), whereas PL GABAergic (PL\textsuperscript{GABA}) neurons are implicated in both the encoding and the retrieval of threat associations by regulating the firing rate of PL\textsuperscript{GLUT} neurons (Courtin et al., 2014; Cummings and Clem, 2020).
in a safe context, food-associated cues activate both \( PL_{GLUT} \) and \( PL_{GABA} \) neurons 
(Burgos-Robles et al., 2013; Gaykema et al., 2014), and inactivation of \( PL_{GLUT} \) neurons 
may increase or reduce conditioned food-seeking responses depending on the specific 
downstream projections that are being modulated (Otis et al., 2017). While these 
studies suggest a role for both \( PL_{GLUT} \) and \( PL_{GABA} \) neurons in the regulation of threat 
and food-seeking responses in isolation, it remains unexplored how these two subsets 
of \( PL \) neurons regulate the trade-off between seeking rewards and avoiding potential 
threats during a conflict situation. To address this question, we combined single-unit 
recordings with optogenetics to track the neuronal activity of photoidentified \( PL_{GLUT} \) and 
\( PL_{GABA} \) neurons during the test session.

For photoidentification of \( PL_{GLUT} \) neurons, we injected into \( PL \) a viral vector (AAV-
CaMKII\( \alpha \)-hChR2-(H134R)-eYFP) with a gene promoter (CaMKII\( \alpha \)) that favors the 
expression of the light-activated cation channel channelrhodopsin (ChR2) in \( PL_{GLUT} \) 
neurons. This CaMKII\( \alpha \) labeling approach has been successfully used in previous 
studies (Gradinaru et al., 2009; Tye et al., 2011) and was validated here for \( PL \) neurons 
by showing a lack of immunocolabeling between the viral vector and the GABAergic 
marker GAD67 (Fig. 5A). Rats expressing ChR2 selectively in \( PL_{GLUT} \) neurons were 
implanted with an optrode into the same region for optogenetic-mediated identification 
of \( PL_{GLUT} \) neurons at the end of the behavioral session (Fig. 5B). Among the recorded 
\( PL \) cells, 39 out of 104 neurons (n = 5 rats) showed short-latency responses (< 6 ms) to 
laser illumination and were classified as \( PL_{GLUT} \) neurons (Fig. 5C-D and Methods).

Previous studies have shown that photoactivation of \( PL_{GLUT} \) neurons can lead to indirect 
activation of synaptically connected neurons in the region, but these indirect responses
to laser illumination in cortical regions take longer than 9 ms to occur (Lima et al., 2009).

For photoidentification of PL$^{\text{GABA}}$ neurons, we injected into PL a viral vector (AAV-mDlx-ChR2-mCherry) with a gene promoter (mDlx) that favors the expression of ChR2 in PL$^{\text{GABA}}$ neurons. This mDlx labeling approach has been successfully used in previous studies (Dimidschstein et al., 2016; Sun et al., 2020), and was validated here for PL neurons by using two different methods: an immunohistochemical approach that resulted in significant immunocolabeling between the viral vector and the GABAergic marker GAD67 (Fig. 5E), and an in situ hybridization approach which confirmed that ~88% of the cells labeled with the viral vector also expressed the GABAergic marker vGAT (Supplementary Fig. 4A-B). Rats expressing ChR2 selectively in PL$^{\text{GABA}}$ neurons were implanted with an optrode into the same region for optogenetic-mediated identification of PL$^{\text{GABA}}$ neurons at the end of the behavioral session (Fig. 5F). Among the recorded PL cells, 84 out of 338 neurons (n = 19 rats) showed short-latency responses to laser illumination (< 12 ms) and were classified as PL$^{\text{GABA}}$ neurons (Fig. 5G-H and Methods). A longer latency criterion was used for GABAergic neurons because indirect photoactivation of neighboring cells would require a disinhibitory mechanism that has been shown to take longer than 15 ms in cortical regions (Pi et al., 2013).

After separating the photoidentified cells into PL$^{\text{GLUT}}$ and PL$^{\text{GABA}}$ neurons, we aligned their activities to the onset of the food cues and compared changes in firing rates from the reward to the conflict phase in Pressers (Fig. 5I). We observed that the proportions of excitatory and inhibitory food cue responses for PL$^{\text{GLUT}}$ and PL$^{\text{GABA}}$ neurons were similar during the reward and the conflict phases (Fig. 5J-K). Next, we
analyzed the spontaneous activity of PL\textsubscript{GLUT} and PL\textsubscript{GABA} neurons and compared
changes in their firing rates across the different phases of the test session (Fig. 5L). We found that the average firing rate of PL\textsubscript{GLUT} neurons remained the same across the
different phases of the test (~5 Hz; Fig. 5M), with most of the cells (53%) changing their
activities in more than one session (Fig. 5N). A group analysis of the firing rates across
phases demonstrated that PL\textsubscript{GLUT} neurons were disinhibited during the odor phase, and
subsequently inhibited during the conflict phase when Pressers resumed searching for
food (Fig. 5O). Similar to PL\textsubscript{GLUT} neurons, the average firing rate of PL\textsubscript{GABA} neurons also
remained the same across the different phases of the test (~8 Hz, Fig. 5P), with most of
the cells (65%) changing their activities in more than one session (Fig. 5Q). However, in
contrast to PL\textsubscript{GLUT} neurons, a group analysis of the firing rates of PL\textsubscript{GABA} neurons did
not reveal significant differences across the phases (Fig. 5R). Because PL is comprised
of different subpopulations of interneurons that inhibit each other during food seeking or
defensive responses (Gaykema et al., 2014; Cummings and Clem, 2020), we cannot
rule out the possibility that distinct subsets of PL\textsubscript{GABA} neurons were preferentially
recruited during each one of the phases.

To evaluate how the spontaneous activity of the same PL neurons changed
during the test session, we tracked the firing rate of PL\textsubscript{GLUT} and PL\textsubscript{GABA} neurons across
the different phases. We found that all PL\textsubscript{GLUT} neurons that were either excited or
inhibited during the reward phase responded in opposite direction or did not change
their activities during the odor phase (Supplementary Figure 5A-B), suggesting the
existence of distinct subpopulations of PL\textsubscript{GLUT} neurons that encode valence-specific
information. In contrast, no significant differences in the proportions of excitation and
inhibition were observed in $PL^{GABA}$ neurons during the transition from reward to odor phase nor during the transition from odor to conflict phase for both subsets of $PL$ neurons (Supplementary Figure 5C-F). Furthermore, both $PL^{GLUT}$ and $PL^{GABA}$ neurons showed the same proportion of excitatory and inhibitory responses during the onset of freezing, avoidance, or risk-assessment behaviors (Supplementary Figure 6A-C), indicating that both subsets of PL neurons may contribute to the expression of distinct defensive responses during conflict. Together, our data suggest that $PL^{GLUT}$ neurons are disinhibited when foraging rats exhibit increased defensive behaviors in response to the conditioned odor and become inhibited again when animals resume searching for food during the conflict phase.

**Putative classification of PL neurons based on spike waveform and spike timing parameters is insufficient to differentiate glutamatergic and GABAergic cells**

Previous single-unit recording studies have used an indirect cluster analysis of spike waveform and spike timing parameters to classify neurons into putative glutamatergic or putative GABAergic cells (Hassani et al., 2009; Courtin et al., 2014; Fan et al., 2017). While it is widely accepted that glutamatergic neurons exhibit longer spike duration and lower firing rate than GABAergic neurons (Mruczek and Sheinberg, 2012; Ono et al., 2017), a direct comparison between optogenetic photoidentification and putative classification of PL neurons *in vivo* is currently missing to confirm that extracellular spike parameters can be reliably used to separate glutamatergic and GABAergic cells.
We sought to address this question by extracting the extracellular spiking properties of the 123 photoidentified PL neurons shown in Figure 5 and plotting their values to perform a cluster analysis separation. Because the amplitude of the waveform has been shown to directly correlate with the distance between the recording electrode and the neuronal soma (Weir et al., 2014), we limited our analyses to the duration of the action potentials (i.e., spike half-width) and the firing rate of the neurons (i.e., frequency), similar to previous putative classifications in vivo (Frank et al., 2001; Constantinidis and Goldman-Rakic, 2002; Bartho et al., 2004). Using an unbiased unsupervised cluster-separation algorithm, we separated the cells into putative PLGLUT and putative PLGABA neurons based on spike waveform and spike timing features by using k-means++ algorithm as a heuristic to find centroid seeds for k-means clustering (see Methods for details). Only in vivo photoidentified GABAergic or glutamatergic neurons were considered for this analysis. The spike half-width feature was the driving force for the classification and indicated a division of clusters at ~0.175 ms (Supplementary Figure 7A). By marking the cells that were previously photoidentified as PLGLUT or PLGABA neurons on the same cluster distribution, we calculated the percentage of PLGLUT or PLGABA neurons that were correctly identified with the putative classification (Supplementary Figure 7A-B). Surprisingly, we observed less than 40% of overlapping between the optogenetic photoidentification and the putative analysis (Supplementary Figure 7B-C), indicating that cell-type putative classification of glutamatergic and GABAergic neurons in PL should be treated with caution. These results are consistent with prior studies in vivo showing that pyramidal and inhibitory neurons exhibit a wide variety of frequencies and spike durations, which alone may not
be sufficient for putative cell-type classification (Vigneswaran et al., 2011; Becchetti et al., 2012; Weir et al., 2014).

Photoactivation of PLGLUT, but not PLGABA neurons, suppresses reward-seeking responses.

To further establish whether changes in the activity of PL neurons can alter cue-triggered food-seeking responses, we used an optogenetic approach to selectively activate either PLGLUT or PLGABA neurons during a cued food-seeking test in a neutral context. We initially infused either the viral vector AAV-CaMKIIα-ChR2-eYFP (Fig. 6A) or AAV-mDlx-ChR2-mCherry (Fig. 6E) into PL and implanted an optrode into the same region to examine how photoactivation of PLGLUT or PLGABA neurons change local activity. Laser illumination of PLGLUT somata increased the firing rate of most responsive PL neurons (9 out of 20 neurons, 45%), with some neurons showing reduced activity (6 out of 20 neurons, 30%, Fig. 6B-D). Neurons that increased their activities showed shorter response latencies (2.1 ±0.24 s) compared to neurons that reduced their activities (41.3 ± 5.57 s), suggesting direct responses (i.e., opsin-mediated) versus indirect responses (i.e., multi-synaptic), respectively. Conversely, laser illumination of PLGABA somata reduced the firing rate of all responsive PL neurons (16 out of 22 neurons, 73%; Fig. 6F-H), indicating a suppression in local activity. After investigating the local effects of photoactivating either PLGLUT and PLGABA neurons, we infused another set of animals with the same viral vectors in PL and implanted bilateral optical fibers into the same region to manipulate PL activity during the cued food-seeking test (Fig. 6I-J). Rats expressing only eYFP in PL were used to control for any nonspecific
effects of viral transduction or laser heating. To assess the effects of PL photoactivation on lever presses, we alternated two trials of food cues with the laser on vs. laser off conditions (Fig. 6K-L). Photoactivation of $PL^{GLUT}$ (CaMKIIα-ChR2), but not $PL^{GABA}$ (mDlx-ChR2) neurons, reduced the frequency of lever presses (Fig. 6M) and increased the latency for the first press after the cue onset (Fig. 6N), when compared to control group. Photoactivation of either $PL^{GLUT}$ or $PL^{GABA}$ neurons did not induce freezing behavior (Fig. 6O). These results are consistent with our electrophysiological recordings in Fig. 5O showing that increased inhibition in the firing rate of $PL^{GLUT}$ neurons correlates with augmented reward-seeking responses during conflict. Overall, these findings suggest that increasing the activity of $PL^{GLUT}$ neurons is sufficient to suppress cued reward-seeking responses in a neutral context.

**Photoinhibition of $PL^{GLUT}$ neurons in Non-pressers reduces freezing responses and increases food approaching during conflict.**

Our electrophysiological experiments in Fig. 5O demonstrate that $PL^{GLUT}$ neurons are disinhibited when rats express defensive responses to the conditioned odor. In addition, our photoactivation experiments in Fig. 6K-O indicate that increasing the activity of $PL^{GLUT}$ neurons suppresses cued reward-seeking behavior in rats that are pressing a lever for food. We next hypothesized that photoinhibition $PL^{GLUT}$ neurons during conflict would attenuate defensive behaviors and rescue food-seeking responses in Non-Pressers. To test this hypothesis, we injected a group of rats with the viral vector AAV-CaMKIIα-eNpHR-eYFP (or AAV-CaMKIIα-eYFP) into PL to express the inhibitory opsin halorhodopsin (or eYFP control) selectively in $PL^{GLUT}$ neurons (Fig. 7A). Rats were
initially exposed to a cued food-seeking test to assess the effects of photoinhibition of PLGLUT neurons on food-seeking responses in a neutral context. We observed that photoinhibition of PLGLUT neurons had no effect on lever pressing rate, latency to press the lever or freezing responses before threat conditioning (Supplementary Fig. 8A-E). Animals were then threat conditioned as in Fig. 1 and on the following day exposed to the odor arena for a test session. During the conflict phase, the first pair of food cues was used to classify the animals into Pressers and Non-Pressers, whereas the subsequent pairs of food cues were alternated between laser on and laser off conditions to assess the effects of illumination of PLGLUT neurons on approach-avoidance responses (Fig. 7B-C). Remarkably, photoinhibition of PLGLUT neurons (CaMKIIα-eNpHR) in Non-Pressers reduced the percentage of time rats spent freezing (Fig. 7D) and avoiding the odor area (Fig. 7E), and increased the percentage of time rats spent approaching the food area (Fig 7F) during the food cue presentation, when compared to the eYFP-control group. Despite the increase in food approaching behavior, photoinhibition of the same cells had no effects on lever pressing responses (Fig. 7G-H). In another subset of Non-Presser rats, photoactivation of PLGABA neurons (mDlx-ChR2) did not alter food seeking nor defensive responses during the conflict phase, when compared to the mCherry-control group (Fig 7I-P). Taken together, these results demonstrate that reduced activity in PLGLUT neurons during conflict situations reduces defensive responses and biases rats’ behavior toward food seeking.
DISCUSSION

Using a novel approach-avoidance conflict test, we demonstrated that PL neurons regulate reward-approach vs. threat-avoidance responses during situations of uncertainty, when rats use previously associated memories to guide their decisions. We found that increased risk-taking behavior in Pressers was associated with a larger number of food-cue responses in PL neurons, which were higher in magnitude and persisted during the conflict phase, when compared to Non-Pressers. In addition, PLGLUT neurons showed reduced spontaneous activity during risky reward seeking and photoactivation of these cells in a neutral context was sufficient to suppress lever press responses. Accordingly, photoinhibition of PLGLUT neurons at the onset of the food cues in Non-Pressers reduced defensive responses and increased food-approaching during the conflict phase, consistent with our observation that a small fraction of PL neurons changed their activity at the onset of freezing, avoidance or risk-assessment responses. Altogether, these results suggest that under memory-based conflict situations, reduced or increased activity in PLGLUT neurons can favor the behavioral expression of food-approaching or threat-avoidance responses, respectively.

During our approach-avoidance conflict test, Pressers and Non-Pressers showed similar levels of lever pressing before the conflict phase (e.g., cued food-seeking training, threat conditioning, and reward phases). This observation suggests that these two individual phenotypes most likely emerged during the test session and were independent of prior differences in sucrose preference or food-seeking motivation. Similarly, because both groups exhibited the same percentage of freezing during the olfactory threat conditioning session, the increased defensive behaviors and the
reduced food-seeking responses observed in Non-Pressers during the test session were unlikely due to higher acquisition of conditioned threat responses. Furthermore, other external factors such as shock sensitivity or pain tolerance cannot be accounted for the individual differences observed in our experiments because both groups reacted equally to the unconditioned stimulus (i.e., velocity measured as maximum speed after the footshocks) and, different from other conflict tasks using footshocks as a punishment during the conflict test (Geller, 1960; Vogel et al., 1971; Oberrauch et al., 2019), in our model rats were not exposed to footshocks during the conflict phase. Therefore, the most plausible interpretation for the behavioral differences observed in our task is that Pressers and Non-Pressers have allocated distinct motivational significance to the food- or shock-paired cues during the test session.

Individual differences in risky decision making have also been reported in other studies using rodent models of behavioral conflict involving footshock punishment (Simon et al., 2009; Jean-Richard-Dit-Bressel et al., 2019; Bravo-Rivera et al., 2021), reversal learning (Bari et al., 2010), or variations in reward probability (Ainslie, 1975; St Onge and Floresco, 2009; Dellu-Hagedorn et al., 2018), although the neural mechanisms underlying such differences are less clear. Evidence indicates that some of the neurobiological bases of individual variation in stimulus-reward response depend on differences in dopamine levels in subcortical circuits (Tomie et al., 2000; Flagel et al., 2007; Flagel et al., 2011), which are regulated by top-down mechanisms involving the mPFC (Ferenczi et al., 2016; Haight et al., 2017; Serrano-Barroso et al., 2019). Accordingly, our neural correlate analyses of risk-taking vs. risk-avoiding behaviors in the PL subregion of the mPFC revealed some clear differences between the two
phenotypes, suggesting that PL neurons participate in behavioral selection when rats’
decision depends on the conflicting memories of reward and threat. One interesting
finding in our study was the observation that Pressers showed a larger number and a
higher magnitude of food-cue responses during the conflict phase when compared to
Non-Pressers, indicating that PL neurons can differentiate between situations involving
motivational conflict and those that do not. Because PL neurons are known for encoding
the value of reward-predictive cues (Sharpe and Killcross, 2015; Otis et al., 2017), the
increase in the number and magnitude of food cue responses observed in Pressers
might result in a greater allocation of attention to food cues, which would explain the
persistent reward-seeking responses observed in this group during motivational conflict.
In support of this interpretation, reward-paired cues can acquire motivational salience in
some subjects and become sufficient to elicit reward-seeking responses in both rodents
(Robinson and Flagel, 2009; Robinson et al., 2014) and humans (Smith et al., 2011;
Jensen and Walter, 2014). Consistently, Pressers also showed a larger number and a
higher magnitude of food cue responses in PL before the conflict phase (i.e., reward
phase), although the percentage of rewarded presses and the latency to press the lever
during the reward phase were similar between the two groups.

Another possible interpretation for the differences in food-cue responses in
Pressers and Non-Pressers is the reduced excitatory-food cue responses in Non-
Pressers, which may be mediated by cue-evoked inhibitory inputs to PL during the
conflict phase. While the source of this inhibition is unclear, a potential candidate are
GABAergic neurons in the ventral tegmental area (VTA^GABA), which correspond to 35%
of the cells in this region and send significant projections to PL (Nair-Roberts et al.,
Previous studies have shown that VTA\textsuperscript{GABA} neurons change their firing rates in response to reward-predicting cues (Cohen et al., 2012), and chemogenetic activation of these cells suppress the activity of local dopaminergic neurons (van Zessen et al., 2012), reduces cue-evoked sucrose-seeking responses (Wakabayashi et al., 2019), and induces conditioned place aversion in rodents (Tan et al., 2012). Future studies need to determine whether this regulation of rewarding and aversive responses by VTA\textsuperscript{GABA} neurons can also be attributed to their long-range inhibitory projections to PL neurons, particularly during conflict situations.

Differences in risk-taking and risk-avoiding behaviors were also reflected on LFP frequencies in PL neurons in the beginning of the test session, with \textit{Pressers} and \textit{Non-Pressers} displaying increased power in the delta or theta bands, respectively. These findings are in corroboration with previous studies showing that increased delta power activity in the mPFC is associated with both reward-seeking and preparatory attention (Horst and Laubach, 2013; Totah et al., 2013; Emmons et al., 2016), whereas augmented theta power in the mPFC or synchronized theta activity between mPFC and BLA is correlated with the expression of avoidance responses or the consolidation of threat memories, respectively (Popa et al., 2010; Padilla-Coreano et al., 2019). More specifically, increased synchrony between mPFC and BLA activity in the theta frequency range has been reported for animals that successfully differentiate between aversive and safe cues (or environments) during a differential threat conditioning task (or an open field arena) (Likhtik et al., 2014; Stujenske et al., 2014). In addition, prior studies have shown that 4 Hz LFP oscillations in the mPFC and BLA were strongly synchronized during conditioned freezing episodes (Courtin et al., 2014; Dejean et al.,...
and these sustained 4 Hz oscillations in the mPFC were independent of hippocampal low-theta oscillations, suggesting that they were internally generated in the mPFC during the expression of freezing behavior (Karalis et al., 2016). Consistent with these findings, in our study Non-Pressers showed increased theta activity and marked 4 Hz oscillations in PL neurons, which were associated with better discrimination between reward and threat cues and increased freezing responses during the test session, when compared to Pressers.

Increased risk-taking behavior in Pressers was associated with a higher number of PLGLUT neurons showing reduced spontaneous activity during the conflict phase. In contrast, risk-avoiding responses in Non-Pressers were associated with increased spontaneous activity during conflict. While this set of results suggest that distinct patterns of PL activity are associated with risk-taking or risk-avoiding behaviors in conflict situations, our optogenetic manipulation provided a causal role for PLGLUT neurons in the regulation of approach-avoidance conflict. For instance, the reduction in food-seeking responses during photoactivation of PLGLUT neurons indicates that increased activity in PL pyramidal cells is sufficient to recapitulate the reward-seeking suppression observed during conflict. Our findings agree with previous studies showing that increased activity in mPFC neurons, including PL, attenuates reward-seeking responses in a neutral context (Berglind et al., 2007; Chen et al., 2013; Ferenczi et al., 2016; but see: Warthen et al., 2016), an effect that has been attributed, at least in part, to downstream projections to the paraventricular nucleus of the thalamus (PVT) (Otis et al., 2017). Notably, PVT neurons are necessary for the retrieval of both reward- and threat-associated memories (for a review see: Do Monte et al., 2016; Millan et al., 2017;
McGinty and Otis, 2020; Penzo and Gao, 2021), and activity in PVT neurons has recently been shown to be associated with the regulation of approach-avoidance responses during situations of conflict (Choi and McNally, 2017; Choi et al., 2019; Engelke et al., 2021), suggesting a potential target by which PL glutamatergic neurons may exert their effects.

Considering that Pressers showed a higher number of excitatory food-cue responses than Non-Pressers, it is counterintuitive that photoactivation of PLGLUT neurons during the food cue onset resulted in reduced food-seeking responses. However, it is important to note that our optogenetic manipulation not only altered the activity of food-cue responsive neurons, but mostly the global activity of other PLGLUT neurons. Thus, it is possible that increased activity in the firing rate of PLGLUT neurons may result in reduced signal-to-noise ratio during the food cue onset (Kroener et al., 2009; McGinley et al., 2015), and consequently decreased food-seeking responses. In contrast, by reducing their spontaneous firing rates during conflict situations, PLGLUT neurons become more likely to fire in response to food cues due to an increase in the signal-to-noise ratio, thereby resulting in persistent reward-seeking responses during the conflict phase as we propose in our schematic in Fig. 8.

Additionally, our findings showing that inactivation of PLGLUT neurons increases food-approaching responses in Non-Pressers suggest that PL activity is indispensable to inhibit reward pursuit in the presence of threat-associated cues. The lack of effects on lever pressing may indicate that other parallel brain regions may be modulating the suppression of operant lever-press responses during conflict. Alternatively, photoinhibition of PLGLUT neurons was not large enough to produce a more global effect.
on risky behavior (i.e., completely restore lever presses). Collectively, these results add
to a growing literature indicating that PL neurons are necessary to guide appropriate
food-seeking behavior in tasks that rely on discrimination among environmental cues
(Marquis et al., 2007; Sangha et al., 2014; Moorman and Aston-Jones, 2015) or
decision-making tasks involving risk of punishment in which animals need to: i) adapt
choice behavior during shifts in risk contingencies (Orsini et al., 2018), ii) regulate
behavioral flexibility (Radke et al., 2015; Capuzzo and Floresco, 2020), or iii) suppress
reward seeking in response to conditioned aversive stimuli (Kim et al., 2017; Piantadosi
et al., 2020). Moreover, our results are in accordance with previous findings
demonstrating that inactivation of PL neurons, or their inputs from BLA, increases risk-
taking behavior in a conflict task in which rats needed to refrain from consuming
sucrose to avoid a footshock (Burgos-Robles et al., 2017; Verharen et al., 2019).

Previous studies have shown that PL neurons fire in response to shock-paired
cues and such activity is highly correlated with the expression of freezing responses
(Burgos-Robles et al., 2009; Sotres-Bayon et al., 2012; Kim et al., 2013; Courtin et al.,
2014). Adding to these findings, our recordings demonstrated that the activity of a small
number of PL neurons correlated with the onset of freezing responses, with the same
proportion of freezing-responsive cells being classified as $\text{PL}^{\text{GLUT}}$ or $\text{PL}^{\text{GABA}}$ neurons
(~10%). At first sight, the lack of effects on freezing behavior following optogenetic
activation of $\text{PL}^{\text{GLUT}}$ neurons seems at odds with our recordings. It also seems to
disagree with previous studies showing that electrical stimulation or optogenetic
induction of 4Hz oscillations in PL increases conditioned freezing responses (Vidal-
Gonzalez et al., 2006; Courtin et al., 2014) by synchronizing the neural activity between
PL and BLA regions (Karalis et al., 2016). However, one important difference between our study and others is that photoactivation of PL\(^{\text{GLUT}}\) neurons in our experiments was performed in naïve rats, in the absence of shock-paired cues. Thus, the increased freezing responses following PL activation reported in previous studies appear to be dependent on the preexistence of a conditioned threat memory.

Overall, our results outline the neural correlates of risk-taking and risk-avoiding behaviors in PL and reveal an important role for PL\(^{\text{GLUT}}\) neurons in coordinating memory-based risky decision-making during conflict situations. Further studies will focus on identifying the PL downstream/upstream circuits that regulate reward-approaching and threat-avoidance responses, as well as the potential genetic and epigenetic factors that could contribute to the observed behavioral phenotypes. Elucidating the underlying mechanisms that mediate risk-taking vs. risk-avoiding responses during situations of uncertainty may help to provide understanding of response selection and adaptive behaviors, and may have clinical relevance to many psychiatric disorders (Aupperle and Paulus, 2010; Kirlic et al., 2017). Whereas persistent avoidance of presumed threats is the cardinal symptom of anxiety disorders (Treanor and Barry, 2017), seeking reward despite negative consequences is a hallmark of both eating and substance use disorders in humans (Volkow et al., 2012).

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**Author contributions**

J.A.F-L., D.S.E., and G.A.M. performed the behaviors, optogenetics, and single-unit recordings. D.S.E. carried out the immunohistochemical and *in situ* hybridization studies. A.G. wrote and validated the DeepLabCut and the Python codes for behavioral analyses. J.A.F-L. wrote the MATLAB and Python codes for single-unit recording analyses. Both J.A.F-L. and F.H.D-M. analyzed the single-unit recording data. D.S.E. performed the statistical analyses. All the authors helped to design the study and interpret the data. J.A.F-L. and F.H.D-M. prepared the manuscript with comments from all the co-authors.

**Competing interests**

The authors declare no competing interests.

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METHODS

Animals. All experimental procedures were approved by the Center for Laboratory Animal Medicine and Care of The University of Texas Health Science Center at Houston. The National Institutes of Health guidelines for the care and use of laboratory animals were strictly followed to minimize any potential discomfort and suffering of the animals. Male Long-Evans hooded adult rats (Charles Rivers Laboratories) with 3-5 months of age and weighing 300-450 g at the time of the experiment were used. Rats were single-housed and after a 3-day acclimation period handled and trained to press a lever for sucrose as described below. Animals were kept in a 12-hour light/12-hour dark cycle (light from 7:00 to 19:00) and maintained on a restricted diet of 18 g of standard laboratory rat chow provided daily at end of experimentation. Animals were given ad libitum access to water. Animals’ weights were monitored weekly to ensure all animals maintained their weight under food restriction. During pre- and post- surgery phases, animals were given ad libitum access to food for a total of 7 days.

Surgeries. Rats were anaesthetized with 5% isoflurane in an induction chamber. Animals were positioned in a stereotaxic frame (Kopf Instruments) and anesthesia was maintained with 2.5% isoflurane delivered through a facemask. A heating pad was positioned bellow the body of the animal and both temperature and respiration were monitored during the entire surgery. Veterinary lubricant ointment was applied on the eyes to avoid dryness during the surgery. Animals received a subcutaneous injection of the local anesthetic bupivacaine (0.25%, 0.3 ml) at the incision site. Iodine and ethanol (70%) were alternately applied for asepsis of the incision site. The surgery procedures varied according to the type of implantation/injection (see below). For injection-only
surgeries, the incision was stitched after the injection by using surgical suture (Nylon, 3-0). For implantation surgeries, the implants were fixed to the skull using C&B metabond (Parkell), ortho acrylic cement, and four to six anchoring screws. After surgery, animals received a subcutaneous injection of meloxicam (1 mg/Kg) and a topical triple antibiotic was applied to the incision area.

**Viral vector injection.** Viral injections were performed using a microsyringe (SGE, 0.5 μl) with an injection rate of 0.04 μl/min plus an additional waiting time of 12 min to avoid back-flow. The adeno-associated virus (AAV) was bilaterally injected at a volume of 0.4 μl per side. The AAV-CaMKIIα-eNpHR-eYFP vector was used to inhibit glutamatergic neurons, whereas AAV-mDlx-ChR2–mCherry or AAV-CaMKIIα-ChR2-eYFP vectors were used to activate either GABAergic or glutamatergic neurons, respectively. The use of mDlx or CaMKIIα promoters enabled transgene expression favoring either GABAergic or glutamatergic neurons, as previously shown (Gradinaru et al., 2009; Tye et al., 2011; Dimidschstein et al., 2016; Sun et al., 2020) and was confirmed by our immunohistochemical and RNAscope assessment (Supplementary Fig. 4). The viral construct AAV-CaMKIIα-eYFP was used to control for any nonspecific effects of viral infection or laser heating. All plasmid or viral vectors were obtained from Addgene or University of North Carolina Viral Vector Core. For implantation of optrodes, the following coordinates from bregma were used for virus injection: PL, +2.7 mm AP, ±0.7 mm ML, -3.8 mm DV at a 0° angle. For PL soma illumination, an optical fiber (0.39 NA, 200 nm core, Inper) was implanted in each hemisphere targeting PL neurons using the following coordinates from bregma: +2.7 mm AP; ±1.5 mm ML; -4.0 mm DV at a 15° angle.
Single-unit electrodes. An array of 16 or 32 microwires was unilaterally implanted targeting the PL using the following coordinates from bregma: +2.7 mm AP, ±0.8 mm ML, -3.9 mm DV. Three different electrode configurations were used: i) 32-channel silicon probes (Buzsaki32-CM32 or A1x32-5mm-25-177-CM32, Neuro Nexus Technologies, USA), ii) Micro-Wire Arrays (MWA) of 16 or 32 channels (Bio-Signal Technologies Ltd, USA); or iii) custom designed electrodes with 2×8 grid with 150 μm of space between wires, 200 μ of space between rows, with 35 μm diameter wires (Innovative Neurophysiology Inc., USA). For photoidentification of GABAergic or glutamatergic neurons, a Hermes 32 channels optrode array was used (200 nm core, Bio-Signal Technologies Ltd). Optrodes were unilaterally implanted at the same coordinates described above after the infusion of 0.6 μl of AAV-mDlx-ChR2–mCherry or AAV-CaMKIIα-ChR2-eYFP vectors. In all cases, the ground wire was wrapped around a grounding screw previously anchored into the skull. Two insulated metal hooks were implanted bilaterally into the cement to allow firmly attachment of the array connector to the cable during recording.

Odor preparation. A 99% amyl acetate solution (Sigma Aldrich) was diluted in propylene glycol (Bluewater Chemgroup, Inc) to a 10% solution and presented to the rats during the different stages and phases of the olfactory threat conditioning test. A customized olfactometer (Med Associates) was used to control the flow of air into the animal’s chamber. Before being mixed with the 10% amyl acetate solution, the air was passed through a desiccant and a charcoal filter to remove any moisture and odors, and was finally rehydrated with distilled water before being delivered into the chamber.
through a thermoplastic PVC-based tube (Tygon) attached to an odor port located in the odor area.

**Behavioral Tasks**

**Lever-press training.** Rats were placed in a plexiglass, standard operant box (34 cm high x 25 cm wide x 23 cm deep, Med Associates), and trained to press a lever for sucrose on a fixed ratio of one pellet for each press. Next, animals were trained in a variable interval schedule of reinforcement that was gradually reduced across the days (one pellet every 15 s, 30 s, or 60 s) until the animals reached a minimum criterion of 10 presses/min. All sessions lasted 30 min and were performed on consecutive days.

Sucrose pellet delivery, variable intervals, and session duration were controlled by an automated system (ANY-maze, Stoelting). Lever-press training lasted approximately one week, after which animals were assigned to surgery or cued food-seeking training. A small number of rats failed to reach the lever press criteria and were excluded from the experiments (< 3%).

**Cued food-seeking training.** Rats previously trained to press a lever for sucrose were trained to learn that each lever press in the presence of an audiovisual cue (tone: 3 KHz, 75 dB; light: yellow, 2.8 W; 30 s duration) resulted in the delivery of a sucrose pellet into a nearby dish. Reward cue conditioning also took place in the standard operant boxes. While the light cue helps to direct the animals toward the lever during the beginning of the training phase, the tone assures that the animals will not miss the presentation during the trial and provides the temporal precision required for single-unit recordings. After ~4 consecutive days of training (24 trials per day, pseudorandom intertrial interval of ~120 s, 60 min session), rats learned to discriminate the food-
associated cue as indicated by a significant increase in press rate during the presence of the audiovisual cues, when compared to the 30 s immediately before the cue onset (cue-off, see Supplementary Fig. 1A). The cued food-seeking training was completed when animals reached 50% of discriminability index (presses during cue-on period minus presses during cue-off period divided by the total number of presses). After the cued food-seeking training was completed, rats with single-unit electrodes were exposed to an additional training session in which the audiovisual cue ceased immediately after the animals pressed the lever and a single sucrose pellet was delivered into the dish. This extra training reduced the rat’s response to a single press and dish entry per cue, thereby enabling us to correlate each food-seeking event with the neuronal firing rate by avoiding overlapping between consecutive events (e.g., lever presses). The single-pellet training took place in the same plexiglass rectangular arena subsequently used for the odor test (40 cm high x 60 cm wide x 26 cm deep, Med Associates, see schematic in Fig. 1A right). The arena consisted of a hidden area (40 cm high x 20 cm wide x 26 cm deep) separated from an open area by a plexiglass division. An 8-cm slot located in the center of the division enabled the animal to transition between both sides of the arena. For behavioral quantification, the open area was subdivided into a center area and a food area (40 cm high x 12 cm wide x 26 cm deep), the latter containing a lever, a dish, and an external feeder similar to the food-seeking operant box.

Habituation day. Animals were placed in the odor arena and exposed to 12 audiovisual cues (30 s duration, pseudorandom inter-trial intervals of between 25-40 s) followed by 10 min of presentation to the neutral odor alone (10% amyl acetate) and an additional

Habituation day.
12 audiovisual cues similar to the first cues but in the presence of the neutral odor delivered in the food area of the arena. Each lever press in the presence of the audiovisual cue resulted in the delivery of a sucrose pellet into the dish, and the audiovisual cue was ceased immediately after the animal pressed the lever.

**Threat conditioning day.** One day after the habituation day, rats were placed in a plexiglass, standard operant box similar to the cued food-seeking training box, but with the grid floor previously attached to a shock generator system. Rats were habituated to one nonreinforced odor presentation (10% amyl acetate, 30 s duration) followed by five odor presentations that coterminated with a foot shock (0.7 mA, 1 s duration, 258-318 s inter-trial intervals). An olfactometer system was used to precisely deliver the odor into the box (see odor preparation session above), whereas an exhaustor system was used to remove the odor from the box during the intertrial intervals. Between each odor presentation, audiovisual cues (30 s duration) signaling the availability of sucrose were presented to the animals. Each lever press during the audiovisual cues resulted in the delivery of a sucrose pellet into the dish. Shock grids and floor trays were cleaned with 70% ethanol between each rat.

**Test day.** One day after the threat conditioning session, rats were returned to the same arena used during the habituation and exposed to the exact same protocol. The first phase of the test session was called **reward phase** and the animals were exposed to 12 food cues. The second phase was called **odor phase** and the animals were exposed to 10 min of conditioned odor (10% amyl acetate) alone. The last phase was called **conflict phase** and the animals were exposed to 12 food cues in the presence of the conditioned odor. In order to press the lever for sucrose during the conflict phase, rats had to
approach the conditioned odor presented in the food area. After the end of the conflict phase, the odor was extracted from the arena with an exhaustor and the floor and walls of the arena were cleaned thoroughly with 70% ethanol solution.

**Behavioral tracking.** Both the standard operant boxes and the testing arenas were equipped with video cameras and a behavior tracking software (ANY-maze, Stoelting) which were used to record the animal’s behavior and control the delivery of sucrose, foot shock, tone, light and odor in the apparatuses. Avoidance responses were characterized by the time spent in the hidden area of the arena. Freezing responses were characterized by the complete absence of movements except those needed for respiration. Risk-assessment responses were characterized by a body stretching movement to peep out toward the food area while in the hidden area and were used as a measure of risk-assessment behavior (Blanchard et al., 2011).

For single-unit recording analyses, the detection of freezing, avoidance and risk assessment behaviors were performed using the open source tool DeepLabCut, a machine learning software that tracks complex patterns of behavior from videos (Mathis et al., 2018). After a video has been analyzed, the data was saved to a .csv file that contained the x and y location of each rat’s body part in pixels, as well as the analysis of the expected accuracy (i.e., likelihood) of the tracked positions across time. After DeepLabCut has calculated the positions and the likelihood, we used three different Python codes to identify each one of the three behaviors. For freezing behavior, the code used DeepLabCut’s position data and determined if the rat was still for more than 500 ms. The animal was considered to be still if the position in question was within 1.05 pixels of each other. For avoidance behavior, the code used DeepLabCut’s position
data to determine the location of the rat in the arena and based on the center of its head
to identify when the animals entered the hidden area of the arena. Finally, for risk
assessment behavior, the code used DeepLabCut’s position data to identify the nose,
ears, center of the head, and spine to determine whether the rat was located in the
hidden area of the arena with its body stretched and the head looking through the open
division of the apparatus. Each of these codes generated a .xlsx file that contained the
onset and the total duration of each behavioral episode. The time onsets for each
behavior were filtered by selecting only the events that lasted more than 1 second and
were not preceded by the same behavior during the previous 6 s (baseline). The final
list of time onsets was entered into the single-unit recording files to create the events
and temporally align them with the neuronal recordings.

**Optogenetic stimulation during behavior.** Bilateral optical cables (200 μm core, 0.37
NA, 2.5 mm ceramic ferrule, Inper) were connected to a blue laser (diode-pumped solid-
state, 473 nm, 150 mW output, OptoEngine) or a yellow laser (diode-pumped solid-
state, 593.5 nm, 300 mW output, OptoEngine) by using a patch cord (200 μm, 0.39 NA,
FC/PC connector, Inper) through a dual rotary joint (200 μm core, Doric lenses). During
the stimulation, the optical cables were coupled to the previously implanted optical
fibers by using a ceramic sleeve (2.5 mm, Precision Fiber Products). An optogenetic
interface (Ami-2, Stoelting) and an electrical stimulator (Master 9, A.M.P. Instruments)
were used to control the onset of the laser, pulse width, train duration, and frequency.
The power density estimated at the tip of the optical fiber was 7-10 mW for illumination
of PL somata (PM-100D, Power Energy Meter, Thor Labs).
**Single-unit recording.** A 64-channels neuronal data acquisition system (Omniplex, Plexon) integrated with a high-resolution video-tracking system (Cineplex, Plexon) was used for electrophysiological recordings from freely behaving animals. Both videos and neuronal recordings were combined within the same file, thereby facilitating the correlation of behavior with neuronal activity. An electrical isolation, Faraday cage was made and connected to the grounding port of the data acquisition system. The system was connected to the head-mounted electrode/optrode by using a digital headstage cable (32 channels, Plexon), a motorized carrousel commutator (Plexon), and a digital headstage processor (Plexon). Rats were habituated to the headstage cable daily for approximately one week before the beginning of the experiments. Extracellular waveforms exceeding a voltage threshold were band-pass filtered (500 - 5,000 Hz), digitized at 40 KHz, and stored onto disk. Automated processing was performed using a valley-seeking scan algorithm and then visually evaluated using sort quality metrics (Offline Sorter, Plexon). Single-units were selected based on three principal components and waveform features such as valley-to-peak and amplitude measurements. A commercial software (NeuroExplorer, NEXT Technologies), Matlab (MathWorks) scripts, and Python scripting in NeuroExplorer were implemented to calculate the spontaneous firing rate and food-cue responses. The spontaneous firing rate was calculated by comparing the frequency of spike trains during the last 30 s of the food-seeking phase, odor phase, or conflict phase against the 30 s prior to the beginning of each session. Food cue responses were calculated by implementing Matlab scripts as Z-scores normalized to 20 pre-cue bins of 300 ms. Neurons showing a Z-score > 2.58 (p < 0.01) during the first two-bins following the onset of the food cues.
were classified as excitatory responses, whereas neurons showing a Z-score < -1.96 (p < 0.05) during the same first two-bins were classified as inhibitory responses. At the end of the recording sessions, a micro-lesion was made by passing anodal current (0.3 mA for 15 s) through the active wires to deposit iron in the tissue. After perfusion, brains were extracted from the skull and stored in a 30% sucrose/6% ferrocyanide solution to stain the iron deposits.

**Photoidentification of PL neurons during recordings.** During neuronal photoidentification, we recorded from rats expressing channelrhodopsin (ChR2) in PL neurons previously implanted with an optrode in the same region. An optical cable connected to a blue laser was attached to the headstage cable and coupled to the previously implanted optical fiber by using a ceramic sleeve. At the end of the behavioral session, 10 trains of 10-s blue laser pulses (5 ms pulse width, 5 Hz) were delivered by a Master-9 programmable pulse stimulator, which also sent flags to the data acquisition system to mark the time of the laser events. Neurons were considered to be responsive to photostimulation if they showed a significant increase in firing rate above baseline (20 ms, Z-score > 3.29, p < 0.001) within the 6 ms (for PLGLUT neurons) or 12 ms (for PLGABA neurons) after laser, similar to previous studies (Lima et al., 2009; Pi et al., 2013; Burgos-Robles et al., 2017; Engelke et al., 2021).

**Optogenetic manipulation of PL neurons during behavior.** During the cued food-seeking test, rats expressing ChR2 or eNpHR in PL were bilaterally illuminated in the same region by using a blue (5 ms pulse width, 5 Hz for CaMKIIα or 20 Hz for mDlx) or a yellow laser (constant illumination), respectively. The laser was activated at cue onset and persisted throughout the entire 30 s of the audiovisual cue presentation. Rats were
exposed to two consecutive cues with laser off followed by two consecutive cues with laser on in a total of 12 cues (pseudorandom inter-trial intervals of between 25-40 s). To assess the effects of PL illumination on rat’s defensive behavior, PL neurons of rats expressing ChR2 or eNpHR were bilaterally illuminated during 6 distinct epochs of 30 s during the odor phase by using a blue (5 ms pulse width, 20 Hz) or a yellow laser (constant illumination), respectively. To assess the effects of PL illumination on food-seeking responses during the conflict phase, rats were exposed to two consecutive cues with laser off followed by two consecutive cues with laser on in a total of 12 cues (pseudorandom inter-trial intervals of between 25-40 s). The laser was activated at cue onset and persisted on until the animal pressed the bar or the 30 s of the audiovisual cue was completed.

**Histology.** Animals were transcardially perfused with KPBS followed by 10% buffered formalin. Brains were processed for histology as previously described (Do-Monte et al., 2013). Only rats with the presence of eYFP or mCherry labeling and the track of the electrode wires or optical fiber tips located exclusively in PL were included in the statistical analyses.

**Immunohistochemistry.** Rats previously infused with AAV-mDlx-ChR2-mCherry or AAV-CaMKIIα-ChR2-eYFP were transcardially perfused with KPBS followed by 10% buffered formalin. Brains were removed from the skull, transferred to a 20% sucrose solution in KPBS for 24 h, and stored in a 30% sucrose solution in KPBS for another 24 h. Next, coronal PL sections (40 μm thick) were cut in a cryostat (CM 1860, Leica), blocked in 20% normal goat serum and 0.3% Triton X-100 in KPBS at room temperature for 1 h. For identification of GABAergic neurons, PL sections were
incubated with anti-GAD67 serum raised in rabbit (1:400; Millipore-Sigma) at 4°C for 48 h. After sections were washed in KPBS for 5 times, sections were incubated with a secondary anti-rabbit antibody (1:200, Alexa Fluor 488 or Alexa Fluor 594, Abcam) for 2 h. Sections were washed with KPBS, mounted in Superfrost Plus slides, and coverslipped with anti-fading mounting medium (Vectashield, Vectorlabs). Images were generated by using a microscope (Nikon, Eclipse NiE Fully Motorized Upright Microscope) equipped with a fluorescent lamp (X-Cite, 120 LED) and a digital camera (Andor Zyla 4.2 PLUS sCMOS).

**In situ hybridization.** Single molecule fluorescent *in situ* hybridization (RNAscope Multiplex Fluorescent Detection Kit v2, Advanced Cell Diagnostics) was used following the manufacturer protocol for fixed-frozen brains sample. Brain samples were sectioned at a thickness of 20 μm in a cryostat (CM1860, Leica). Sections were collected onto superfrost plus slides (Fisher Scientific) and transferred to a −80°C freezer. To prepare for the assay, brain sections were serially dehydrated with EtOH (50%, 75%, and 100%, each for 5 min) and then incubated in hydrogen peroxide for 10 min. Target retrieval was performed with RNAscope target retrieval reagents at 99°C for 5 min. The sections were then pretreated with Protease III (RNAscope) for 40 min at 40°C. RNAscope probes (Advanced Cell Diagnostics) for mCherry (Cat No. 431201-C3) and vGAT (Cat No. 424541) were hybridized at 40°C for 2h, serially amplified, and revealed with horseradish peroxidase, Opal Dye/TSA Plus fluorophore (Akoya Biosciences), and horseradish peroxidase blocker. Sections were cover-slipped with anti-fading mounting medium with DAPI (Vectashield, Vectorlabs) and kept in the refrigerator. Images were generated by using an epifluorescent microscope (Nikon, Eclipse NiE Fully Motorized
Upright Microscope) equipped with a fluorescent lamp (X-Cite, 120 LED) and a digital camera (Andor Zyla 4.2 PLUS sCMOS). Expression of mCherry mRNA (red, Opal 620) and GAD67 mRNA (green, Opal 520) was determined by using an automated fluorescent threshold detector (NIS-Elements). Colabeled cells were manually counted by an experimenter by measuring either the percentage of mCherry positive neurons in PL that were also labeled with GAD67, or the percentage of GAD67 positive neurons in PL that were also labeled with mCherry.

**Data analyses**

*Behavioral quantification and statistical analysis.* Rats were recorded with digital video cameras (Logitech C920) and behavioral responses were measured by using an automated video-tracking system (ANY-maze) or machine learning (DeepLabCut). Presses per minute were calculated by measuring the number of presses during the 30 s cue multiplied by two. All graphics and numerical values reported in the figures are presented as mean ± s.e.m. Statistical significance was determined with paired or unpaired Student’s t test, repeated-measures ANOVA followed by Bonferroni post-hoc comparisons (Prism 7), and Z-test or Fisher’s exact test, as indicated in Table 1S. Sample size was based on estimations by power analysis with a level of significance of 0.05 and a power of 0.9.

*Single-unit analyses.* Based on data from NeuroExplorer, waveform data and spike timestamps for firing rates was processed through Matlab scripting. The principal-component scores for unsorted waveforms were computed and plotted in a three (or two) dimensional principal-component space. Clusters containing similar valid waveforms were manually defined. After manually clustering similar valid waveforms, a
group of spikes were considered from a single neuron if the waveforms formed a
discrete, isolated, cluster in the principal-component space. The separation of putative
PLGLUT neurons and putative PLGABA neurons was made through an unbiased
unsupervised cluster-separation algorithm based on two electrophysiological properties:
neuron’s average half-spike width (ms) and firing rate (Hz) (Frank et al., 2001). Only in
vivo photoidentified PL neurons (i.e., GABAergic or glutamatergic) were considered for
these analyses. To separate putative PLGLUT neurons and putative PLGABA we used an
unsupervised cluster algorithm based on Matlab’s k-means++ algorithm for centroid
initialization and squared Euclidean distance (Arthur and Vassilvitskii, 2007). The
Euclidian distance was calculated between all neuron pairs to detect 2 clusters on the
two-dimensional space defined by each cluster representing putative GABAergic or
glutamatergic neurons.

Data availability

All the data that support the findings presented in this study are available from the
corresponding author on reasonable request.
REFERENCES


Sierra-Mercado D, Padilla-Coreano N, Quirk GJ (2011) Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. Neuropsychopharmacology 36:529-538.


**Figure 1.** Rats show individual variability in reward-seeking responses during an approach-avoidance conflict test. (A) Schematic and timeline of the approach-avoidance conflict test. (B-D) Rats exhibited an increase in the percentage of time freezing ($F(3.117, 96.63) = 30.4, p < 0.0001$) and a reduction in lever presses ($F(3.771, 116.9) = 11.59, p < 0.0001$) with a higher latency to press the lever ($F(3.29, 102) = 13.09, p < 0.0001$) during the olfactory threat conditioning session on day 1 ($n = 32$), when compared to before the shock. (E-G) Patterns of defensive responses and food seeking during the different phases (reward, odor, conflict) of the test session on day 2. Rats showed an increase in defensive responses characterized by an augment in the percentage of time exhibiting (E) freezing ($F(1.678, 52.01) = 16.56, p < 0.0001$), (F) avoidance ($F(1.85, 57.36) = 38.99, p < 0.0001$) and (G) risk-assessment ($F(1.367, 42.38) = 17.41, p = 0.014$); and a decrease in the (H) percentage of time spent in the food area ($F(1.63, 50.52) = 23.81, p < 0.0001$) during the odor presentation, when compared to the reward phase. Rats’ defensive responses were significantly attenuated during the conflict phase as evidenced by a reduction in the percentage of time (F) avoiding the odor ($p = 0.0031$) and an increase in the percentage of time (H) approaching the food area ($p < 0.0001$), when compared to the odor phase. (I-J) Two different behavioral phenotypes emerged during the conflict phase: rats that continued to press the lever (**Pressers**, green circle, $n = 25$) and rats that showed a complete suppression in lever pressing (**Non-pressers**, purple circle, $n = 7$). The % of rewarded presses was calculated as the percentage of the 12 cue trials in which rats pressed the lever. (K-L) Representative tracks and heatmaps of time spent in each compartment of the arena for a (K) **Presser** or a (L) **Non-Pressers** rat during the test session. (M-R) Patterns of defensive responses and food seeking during the different phases (reward, odor, conflict) of the test session on day 2 after separating the animals into **Pressers** and **Non-pressers**. When compared to **Non-Pressers**, **Pressers** showed reduced defensive responses characterized by an attenuation in the percentage of time exhibiting (M) freezing ($F(2, 60) = 29.54, p < 0.0001$) and (N) avoidance responses ($F(2, 60) = 23.27, p < 0.0001$), and an augment in the percentage of time (P) approaching the food area ($F(2, 60) = 22.49, p < 0.0001$) during both the odor and the conflict phases. Data shown as mean ± SEM. One-way or two-way ANOVA repeated measures followed by Tukey’s or Bonferroni’s multiple comparisons tests, all * $p$’s < 0.05 compared to the same group during the reward phase, all & $p$’s < 0.05 compared to the same group during the odor phase, all $ $ $p$’s < 0.05 compared to **Pressers** during the same phase. All statistical analysis details are presented in table S1. See also Supplementary Fig. 1 and Supplementary Video 1.
FIGURE 2

A Single-unit recordings

n = 32 rats

B Day 2 - Test

Pressers

Non-pressers

C Changes in food cue responses

D Changes in food cue responses

E Excitatory food cue responses

F Excitatory food cue responses

G Inhibitory food cue responses

H Inhibitory food cue responses

I Inhibitory food cue responses

J Inhibitory food cue responses

K Excitatory food cue responses

L Excitatory food cue responses

M Excitatory food cue responses

N Excitatory food cue responses

O Inhibitory food cue responses

P Inhibitory food cue responses

Q Inhibitory food cue responses

R Inhibitory food cue responses

S Inhibitory food cue responses

T Inhibitory food cue responses

U Inhibitory food cue responses

V Inhibitory food cue responses

Selected during reward

Same cells during conflict

n = 48

n = 10

n = 237 neurons

n = 89 neurons

n = 32 rats

n = 31

n = 9
Figure 2. PL neurons respond differently to reward cues in Pressers vs. Non-Pressers during conflict. (A) Diagram of the electrode placements in PL. (B) Schematic and timeline of PL recordings for food cue responses during of the approach-avoidance conflict test (12 food cues per phase). (C) Pie charts showing changes in PL firing rate in response to food cues during reward (left) vs. conflict (right) phases for Pressers (n = 237 neurons from 25 rats, Fisher’s exact test, excitatory during reward phase: n = 48, excitatory during conflict phase: n = 25, p = 0.0049; inhibitory during reward phase: n = 31, inhibitory during conflict phase: n = 7, p < 0.0001. (D) Heatmap of Z-scored neural activities for PL neurons selected during reward phase and tracked to conflict phase. (E) Average peri-stimulus time histograms (PSTHs) for all PL neurons showing excitatory food cue responses (Z-score > 2.58, dotted line) during reward (red line) compared to the same cells during conflict (gray line). (F) Differences in the positive area under the curve (AUC) between the two phases (Wilcoxon test, W = -824, excitatory responses reward phase vs. conflict phase, p < 0.0001). (G-H) Representative PSTHs for a PL neuron showing excitatory responses to food cues during the (G) reward phase vs. the same neuron during the (H) conflict phase. (I) Average PSTHs for all PL neurons showing inhibitory food cue responses (Z-score < -1.96, dotted line) during reward (blue line) compared to the same cells during conflict (gray line). (J) Differences in the negative AUC between the two phases (Wilcoxon test, W = 367, excitatory responses reward phase vs. conflict phase, p < 0.0001). (K-L) Representative PSTHs for a PL neuron showing inhibitory responses to food cues during the reward phase (K) vs. the same neuron during the conflict phase (L). (M) Pie charts showing changes in PL firing rate in response to food cues during reward (left) vs. conflict (right) phases for Non-Pressers (n= 89 neurons from 7 rats; Fisher’s exact test, excitatory during reward phase: n = 10, excitatory during conflict phase: n = 4, p = 0.1620; inhibitory during reward phase: n = 9, inhibitory during conflict phase: n = 2, p = 0.0573). (N-O) Same as D-E, but for Non-Pressers. (P) Differences in the positive AUC between the two phases (Wilcoxon test, W = -37, excitatory responses reward phase vs. conflict phase, p = 0.032). (Q-S) Same as G-I, but for Non-Pressers. (T) Differences in the negative AUC between the two phases (Wilcoxon test, W = -3, excitatory responses reward phase vs. conflict phase, p = 0.455). (U-V) Same as K-L, but for Non-Pressers. Legend: cc, corpus callosum; CG1, anterior cingulate cortex; IL, infralimbic cortex. All statistical analysis details are presented in table S1. See also Supplementary Figs. 2 and 3.
FIGURE 3

Changes in PL responses during freezing

Firing rate 6 s baseline → Firing rate 600 ms after freezing onset

% of neurons

Excited ▶️
Inhibited ▶️
No change ▶️

n = 253 neurons

Changes in PL responses during avoidance

Firing rate 6 s baseline → Firing rate 600 ms after avoidance onset

% of neurons

Excited ▶️
Inhibited ▶️
No change ▶️

n = 256 neurons

Changes in PL responses during risk-assessment

Firing rate 6 s baseline → Firing rate 600 ms after risk-assessment onset

% of neurons

Excited ▶️
Inhibited ▶️
No change ▶️

n = 173 neurons

Percentage of responsive neurons by behavior

Total of responsive neurons

33% (29 out of 88)

n = 253 neurons

Pre-freeze

Non-freeze

Avoidance onset

Risk-assessment onset

Z-Score

1.5 1 0.5 0 0.5 1 1.5

Selected at freezing onset

Same cells during avoidance

Same cells during risk-assessment

Selected at avoidance onset

Same cells during risk-assessment

Selected at risk-assessment onset

Time from behavior onset (s)

-6 0 6 -6 0 6 -6 0 6 -6 0 6
Figure 3. PL activity correlates with the onset of freezing, avoidance or risk-assessment behaviors in both Pressers and Non-Pressers. (A-C) Both Pressers and Non-Pressers showed the same number and proportion of excitatory and inhibitory PL responses during the onset of (A) freezing (Fisher’s exact test, responsive neurons in Pressers: 22 neurons, in Non-Pressers: 15 neurons, p = 0.462), (B) avoidance (Fisher’s exact test, responsive neurons in Pressers: 43 neurons, in Non-Pressers: 9 neurons, p = 0.999) or (C) risk-assessment (Fisher’s exact test, responsive neurons in Pressers: 12 neurons, in Non-Pressers: 6 neurons, p = 0.318) behaviors. (D-F) Representative PSTHs for distinct PL neurons showing excitatory responses at the onset of (D) freezing, (E) avoidance or (F) risk-assessment behaviors. (G-I) Representative PSTHs for distinct PL neurons showing inhibitory responses at the onset of freezing (G), avoidance (H) or risk-assessment (I) behaviors. (J) Venn Diagram showing the percentage of all PL responsive neurons (29 out of 88 neurons) by behavior. Most of the responsive neurons responded exclusively at the onset of one of the behaviors. (K-M) Heatmap of Z-scored neural activities for PL neurons selected at the onset of freezing (K), avoidance (L) or risk-assessment behavior (M) with the same cells tracked during the other behaviors. The threshold used to identify significant differences per neurons was Z-score > 2.58 for excitation and Z-score < -1.96 for inhibition. All statistical analysis details are presented in table S1.
Figure 4. **Pressers and Non-Pressers show significant differences in PL oscillations during the test session.** (A) Power spectral density (PSD) contribution at different frequency bands. (B-C) Average of PSD (%) in the (B) delta (0-4 Hz) or (C) theta (4-10 Hz) bands in *Pressers* (green line, n = 25 rats) and *Non-Pressers* (purple line, n = 7 rats) during the (left) reward, (center) odor, and (right) conflict phases of the test session. *Pressers* showed increased power in the delta band, whereas *Non-Pressers* showed increased power in the theta band during the three phases of the test session (Unpaired Student’s t-test comparing *Pressers* vs. *Non-Pressers*, All p’s < 0.0001). (D-E) Representative time-frequency spectrogram showing changes in the log of PSD (dB) for delta and theta bands in (D) *Pressers* and (E) *Non-Pressers* across the different phases of the session. All statistical analysis details are presented in table S1.
FIGURE 5

Changes in food cue responses

Changes in spontaneous firing rate of PLGLUT neurons

Changes in spontaneous firing rate of PLGABA neurons
Figure 5. In pressers, PLGLUT neurons show reduced spontaneous activity during the conflict phase. (A) Top, Schematic of viral infusion. Bottom, Representative immunohistochemical micrograph showing lack of colabeling (white arrows) between the ChR2 viral construct (green, AAV-CaMKII-ChR2-eYFP) and the GABA marker GAD67 (red), confirming that the use of a CaMKII promoter enables transgene expression favoring PL glutamatergic neurons. Scale bars: 25 µm. (B-D) Photoidentification of PLGLUT neurons. (B) Frequency histogram showing the latency of response to laser illumination for PL neurons (n = 39 photoidentified PLGLUT neurons out of 104 recorded cells). (C) Cells with photoresponse latencies < 6 ms were classified as PLGLUT neurons (Z-score > 3.29, p < 0.001, red dotted line, see details in Methods). (D) Raster plot and peristimulus time histogram showing a representative PLGLUT neuron responding to a 5 Hz train of laser stimulation. (E) Top, Schematic of viral infusion. Bottom, Representative immunohistochemical micrograph showing colabeling (white arrows) between the ChR2 viral construct (red, AAV-mDlx-ChR2-mCherry) and the GABA marker GAD67 (green), confirming that the use of a mDlx promoter enables transgene expression favoring PL GABA neurons. Scale bars: 25 µm. (F-H) Photoidentification of PLGABA neurons. (F) Frequency histogram showing the latency of response to laser illumination for PL neurons (n = 84 photoidentified PLGABA neurons out of 338 recorded neurons). (G) Cells with photoresponse latencies < 12 ms were classified as PLGABA neurons (Z-score > 3.29, p < 0.001, red dotted line, see details in Methods). (H) Raster plot and peristimulus time histogram showing a representative PLGABA neuron responding to a 5 Hz train of laser stimulation. Vertical blue bars: laser onset. Bins of 1 ms. (I) Timeline of PL recordings for food cue responses in pressers during test (12 food cues per phase). (J-K) Stacked bar showing the percentage of (J) PLGLUT neurons or (K) PLGABA neurons that changed their firing rates in response to food cues from the reward phase to the conflict phase. No significant differences were observed across the phases (Fisher’s exact test, all p’s > 0.05; n.s. = non-significant). (L) Timeline of PL recordings for spontaneous activity in pressers during test. (M) Average firing rate of PLGLUT neurons across the different phases of test. (N) Venn diagram showing the percentage of responsive PLGLUT neurons (32 out of 39 neurons) by events. (O) Stacked bar showing the percentage of PLGLUT neurons that changed their spontaneous firing rates across the different phases of the test. PLGLUT neurons were disinhibited during the odor phase (Fisher’s exact test, inhibited in Reward phase: 12 neurons, inhibited in Odor phase: 4 neurons, p = 0.047) and subsequently inhibited during the conflict phase (Fisher’s exact test, inhibited in Odor phase: 4 neurons, inhibited in Conflict phase: 15 neurons, p = 0.0073). (P) Average firing rate of PLGABA neurons across the different phases of test. (Q) Venn diagram showing the percentage of responsive PLGABA neurons (69 out of 84 neurons) by events. (R) Stacked bar showing the percentage of PLGABA neurons that changed their spontaneous firing rates across the different phases of the test. No significant differences were observed across the phases (Fisher’s exact test, all p’s > 0.05; n.s. = non-significant). All statistical analysis details are presented in table S1. See also Supplementary Figs. 4, 5, 6 and 7.
FIGURE 6

A Single-unit recordings from PLGLUT neurons

B Photoactivation of PLGLUT neurons

C Excited neurons

D Laser On

E Single-unit recordings from PLGABA neurons

F Photoactivation of PLGABA neurons

G Inhibited neurons

H Laser On

I CaMKII-ChR2–eYFP

J mDlx-ChR2–mCherry

K Cued sucrose seeking

L Schematic of photoactivation

M Press MGM:

N Laser x:

O Freezing %:
Figure 6. Photoactivation of PLGLUT, but not PLGABA, neurons suppresses reward seeking in a neutral context. (A) Schematic of viral infusion and recordings in PL. (B) Changes in PL firing rate with illumination of PLGLUT neurons in rats expressing AAV-CaMKII-ChR2-eYFP in PL (n= 20 neurons). (C) Average PSTH of PL neurons that were excited during laser illumination of PLGLUT neurons. (D) Raster plot and peri-stimulus time histogram (PSTH) of representative PL neuron showing excitatory responses to illumination in rats expressing AAV-CaMKII-ChR2-eYFP in PL. (E) Schematic of viral infusion and recordings in PL. (F) Changes in PL firing rate with illumination of PLGABA neurons in rats expressing AAV-mDlx-ChR2-mCherry in PL (n= 22 neurons). (G) Average PSTH of PL neurons that were inhibited during laser illumination of PLGABA neurons. (H) Raster plot and PSTH of representative PL neuron showing inhibitory responses to illumination in rats expressing AAV-mDlx-ChR2-mCherry in PL. (I-J) Representative micrograph showing the expression of (I) CaMKII-ChR2-eYFP or (J) mDlx-ChR2-mCherry in PL and schematic of optical fiber location (gray dots) in the same region (compressed across different antero-posterior levels of PL). Green or red areas represent the minimum (dark) and the maximum (light) viral expression into the PL. (K-L) Schematic and timeline of PL photostimulation during the cued-sucrose seeking test in a neutral context. (M-N) Optogenetic activation of PLGLUT neurons (CaMKII-ChR2, dark red circles, n= 7), but not PLGABA neurons (mDlx-ChR2, gold circles, n= 17), reduced the (M) frequency of bar presses (F(10, 180) = 7.009, p<0.0001, CaMKII-ChR2 OFF vs ON Tukey post hoc, p < 0.0001) and increased (N) the latency for the first press (F(10, 180) = 9.931, p < 0.0001, CaMKII-ChR2 OFF vs ON Tukey post hoc, p < 0.0001) when compared to the control group (eYFP-control virus, gray circles, n = 15). (O) Optogenetic activation of PL neurons did not alter freezing behavior (F(10, 180) = 1.124, p = 0.3463). Blue shaded area represents laser-on trials (PLGLUT: 5 Hz, PLGABA: 20 Hz; 5ms pulse width,7-10 mW, 30 s duration). Data shown as mean ± SEM. Each circle represents the average of two consecutive trials. Two-way repeated-measures ANOVA followed by Bonferroni post hoc test. All * p’s < 0.05. All statistical analysis details are presented in table S1.
FIGURE 7

A

Photoinhibition of PLGLUT neurons

AAV-CaMKIIα-eNpHR–eYFP

B

Day 2- Test (Non-pressers)

C

Schematic of photoinhibition

D

Control (n= 5)

CaMKII-eNpHR (n= 11)

E

Avoidance (%)

F

Time in food area (%)

G

Rewarded presses (%)

H

Latency to press (s)

I

Photoactivation of PLGABA neurons

AAV-mDlx-ChR2–mCherry

J

Day 2- Test (Non-pressers)

K

Schematic of photoactivation

L

Control (N= 4)

mDlx-ChR2 (N= 7)

M

Avoidance (%)

N

Time in food area (%)

O

Rewarded presses (%)

P

Latency to press (s)
Figure 7. Photoinhibition of PL\textsuperscript{GLUT} neurons during conflict reduces freezing and increases food approaching in Non-Pressers. (A) Schematic of AAV-CaMKII-eNpHR–eYFP virus infusion in PL and location of optical fibers (gray dots) in the same region (compressed across different antero-posterior levels of PL). Green areas represent the minimum (dark) and the maximum (light) viral expression into the PL. (B-C) Schematic and timeline of the approach-avoidance conflict test during optogenetic inactivation of PL\textsuperscript{GLUT} neurons. (D-H) Photoinhibition of PL\textsuperscript{GLUT} neurons (CaMKII-eNpHR, red bars, n = 11) during the conflict test reduced the percentage of time rats spent (D) freezing (Wilcoxon test, W = -64, laser OFF vs. laser ON, p = 0.0020) and (E) avoiding the odor area (Wilcoxon test, W = -21, laser OFF vs. laser ON, p = 0.031), and increased the percentage of time rats spent in the (F) food area (Wilcoxon test, W = -31, laser OFF vs. laser ON, p = 0.031) during the conflict test without altering (G) the number of lever presses (Wilcoxon test, W = 6, laser OFF vs. laser ON, p = 0.250) and (H) the latency to press (Wilcoxon test, W = -10, laser OFF vs. laser ON, p = 0.125), when compared to controls (eYFP-control virus, white bars, n= 5, Wilcoxon test, Freezing: W = 3, p = 0.812, avoidance: W = 3, p = 0.500, food area: W = 3, p = 0.500, lever presses: W = -1, p = 0.999, latency to press: W = 1, p = 0.999). (I) Schematic of AAV-mDlx-ChR2–mCherry virus infusion in PL and location of optical fibers (gray dots) in the same region (compressed across different antero-posterior levels of PL). Red areas represent the minimum (dark) and the maximum (light) viral expression into the PL. (J-K) Schematic and timeline of the approach-avoidance conflict test during optogenetic activation of PL\textsuperscript{GABA} neurons. (L-P) Photoactivation of PL\textsuperscript{GABA} neurons during the conflict test did not alter rats’ behavior (mDlx-ChR2, gold bars, n= 7, Wilcoxon test, Freezing: W = 18, p = 0.156, avoidance: W = 4, p = 0.500, food area: W = -2, p = 0.750, lever presses: W = 3, p = 0.500, latency to press: W = -3, p = 0.500), when compared to controls (eYFP-control virus, white bars, n = 4, Wilcoxon test, Freezing: W = -2, p = 0.875, avoidance: W = 0, p = 0.999, food area: W = 3, p = 0.500, lever presses: W = 1, p = 0.500, latency to press: all animals reached maximum latency). PL neurons were illuminated from cue onset until the animals pressed the bar or from cue onset until the end of the 30s cues if the animals didn’t press the bar (PL\textsuperscript{GLUT}: 5 Hz, PL\textsuperscript{GABA}: 20 Hz; 5ms pulse width,7-10 mW). Data shown as mean ± SEM. Each bar represents the average of six trials alternated in blocks of 2. Note that the number of data points may appear fewer than the number of rats per group because multiple animals showed the same values. Two-way ANOVA repeated measures followed by Bonferroni post hoc test. All * p’s < 0.05. All statistical analysis details are presented in table S1. See also Supplementary Fig 8.
FIGURE 8

Increased food-seeking responses during conflict

Food-cue responses

Spontaneous firing rate

Reduced food-seeking responses during conflict

Food-cue responses

Spontaneous firing rate

Pressers

Non-Pressers

PL activity

Time

Increased food-seeking responses during conflict

Reduced food-seeking responses during conflict

Lever

Lever
Figure 8. Schematic showing differences in food-cue responses and spontaneous firing rate of PL neurons in *Pressers* and *Non-Pressers*. Left, *Pressers* showed reduced spontaneous firing rate and increased food-cue responses in PL neurons during the conflict test, which resulted in higher signal-to-noise ratio and increased food-seeking responses. Right, *Non-Pressers* showed increased spontaneous firing rate and reduced food-cue responses in PL neurons during the conflict test, which resulted in lower signal-to-noise ratio and reduced food-seeking responses.