**Neural signatures of opioid-induced risk-taking behavior in the prelimbic prefrontal cortex**

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## ABSTRACT

Opioid use disorder occurs alongside impaired risk-related decision-making, but the underlying neural correlates are unclear. We developed a novel approach-avoidance conflict model using a modified conditioned place preference paradigm to study neural signals of risky opioid seeking in the prefrontal cortex, a region implicated in executive decision making. Upon establishment of morphine conditioned place preference, rats underwent a subsequent conflict test in which fear-inducing cat odor was introduced in the previously drug-paired side of the apparatus. While the saline control group avoided the cat odor side, the morphine group maintained preference for the paired side despite the presence of cat odor. K-means clustering identified two subsets of morphine-treated rats that exhibited either persistent drug seeking (Risk-Takers) or increased avoidance (Risk-Avoiders) during conflict. Single-unit recordings from the prelimbic cortex (PL) revealed decreased neuronal firing rates upon acute morphine exposure in both Risk-Takers and Risk-Avoiders, but this firing rate suppression was absent after repeated administration. Risk-Avoiders also displayed distinct post-morphine excitation in PL which persisted across conditioning. During the preference test, subpopulations of PL neurons in all groups were either excited or inhibited when rats entered the paired side. Interestingly, while this inhibitory signal was lost during the subsequent conflict test in both saline and Risk-Avoider groups, these inhibitory responses persisted in Risk-Takers. Our results suggest that loss of PL inhibition after opioid conditioning is associated with the formation of contextual reward memory. Furthermore, persistent PL inhibitory signaling in the drug-associated context during conflict may underlie increased risk taking following opioid exposure.

## INTRODUCTION

Studies in humans have shown a link between harmful patterns of opioid use and deficits in risk-related decision making [1,2], which could partially explain why subjects continue

to pursue opioids despite their apparent dangers. Aligned with this idea, current diagnostic criteria for substance use disorders include the domain of “risky use,” defined as persistent drug intake despite negative consequences or repeated drug use in physically hazardous (risky) situations [3]. However, while many studies have focused on elucidating the biological mechanisms involved in reward-seeking responses during neutral conditions [4,5], relatively little is known about drug seeking in risky circumstances.

To investigate potential causality between opioid use and increased risky decision making, prior studies have attempted to model risky opioid use in laboratory animals. Results have shown that rodents will continue to seek opioids even when they must risk the threat of painful stimuli (e.g., electrical footshocks) to do so [6–13]. Other studies have made further attempts to model the use of opioids in a risky context by introducing an electrified barrier that animals had to cross to obtain the drug [14–19]. However, experimental use of painful stimuli as impediments to drug seeking does not fully resemble the harms associated with obtaining or using drugs in humans [20]. The use of nociceptive stimuli, such as footshock, to investigate risky opioid seeking is further complicated by the fact that repeated opioid use can alter nociceptive sensitivity [21,22], thereby resulting in confounding findings. Additionally, while operant drug self-administration models are important for understanding motivated consumption of drugs, they lack utility in isolating how environmental cues that are inherently tied to drug reward memory affect drug-seeking behavior [23,24].

One animal model that has been extensively used to assess the role of context associations in reward-related behavior is the conditioned place preference (CPP) paradigm [25,26]. Although CPP limits interpretations regarding drug *taking*, some motivational aspects of drug *seeking* are visibly present in that the animal must “choose” to enter and remain in a drug-paired context rather than a neutral one [24]. In addition, contextual drug memories, as measured by CPP, are directly linked to drug craving and are believed to be important drivers of

cue-induced relapse to drug use in humans [23,24,27,28]. With its ability to capture motivated behaviors related to drug reward memory in the absence of drug, CPP is an ideal model for studying drug seeking as a function of environmental context. Thus, to define the phenotype of opioid-induced risk-taking behavior in rats, we developed an approach-avoidance conflict model that pits contextual drug memory against predator odor-induced fear by exposing rats to CPP conditioning with opioids followed by the introduction of a non-nociceptive aversive stimulus (*i.e.,* cat saliva) in the drug-associated context [29,30].

Despite developments in modeling risky drug-seeking behavior in recent years, a complete understanding of the underlying neural mechanisms remains elusive [31,32]. The medial prefrontal cortex (mPFC) is believed to mediate top-down cognitive control of emotionally motivated behavior in humans, but its function in risky reward seeking has not been fully characterized [33]. In rodents, neurons in the prelimbic subregion (PL) of the mPFC are activated by cues that predict either rewarding or threatening stimuli [34–39]. Additionally, aberrant activity in PL has been implicated in the dysregulation of goal-directed behavior and the persistence of drug seeking despite aversive consequences [31,40,41], making this region a potential candidate to regulate risky decision-making. Of particular relevance to opioid seeking, activation of opioid receptors in PL neurons is necessary for the formation of contextual opioid reward memory [42], and the expression of this opioid-associated memory is blocked by chemogenetic silencing of PL [43]. We therefore hypothesized that signaling of drug-related contextual information in PL is suppressed in the presence of threat, and that failure of this suppression occurs during risky opioid seeking. To further investigate the prefrontal cortex mechanisms underlying opioid-induced risky decision making, we performed *in vivo* electrophysiological recordings from PL neurons in freely moving rats to assess changes in PL activity during the development of opioid reward memory, as well as to identify patterns of contextual representation in PL neurons during our opioid-approach versus predator threat-

avoidance conflict model. Our results unravel prefrontal neural correlates of opioid-induced risk-taking choice in a drug-associated context, thereby providing a clearer picture of how substance use alters motivated behaviors.

## MATERIALS AND METHODS

### Animals

Adult male and female Long-Evans hooded rats (Chares Rivers Laboratories) were used. All animals were 3-5 months of age and weighed ~350 to 500 g at the time of testing. Rats were maintained on a 12-h light / 12-h dark cycle (7:00 to 19:00 light period) with *ad libitum* access to water. Rats were also maintained on a restricted diet of standard laboratory rat chow (18 g per day) and weighed weekly to ensure all animals maintained their weights throughout the course of experimentation. All experiments were approved by The University of Texas Health Science Center at Houston Center for Laboratory Animal Medicine and Care. The National Institutes of Health Guide for the Care and Use of Laboratory Animals was followed in order to prevent unnecessary animal suffering or discomfort.

### Drugs

All drugs used in behavioral experiments were injected subcutaneously. Only pharmaceutical-grade morphine sulphate (10 mg/mL, Hikma) or fentanyl citrate (2,500 mcg/50mL, West-Ward) prepared for human intravenous use were administered to animals during the course of the study.

More information about all experimental methods including cat odor collection and preparation, behavioral tasks, stereotaxic surgeries, in vivo single-unit electrophysiology, histology, statistics and reproducibility are presented in detail in the **Supplementary Methods** section.

## RESULTS

### Morphine conditioning leads to contextual place preference and individual differences in risk-taking behavior in rats

To create a model for studying opioid-induced risk-taking behavior, we modified a traditional CPP protocol to include a component of approach-avoidance conflict. First, male adult rats were conditioned in a two-chamber apparatus with systemic administration of either morphine (10 mg/kg, s.c.) or saline in alternating sides every other day for 10 days. All rats received five drug pairings in one side of the apparatus (drug-paired side) and five saline pairings in the other (neutral side; **Fig. 1A**). A control group (saline-treated rats) were subjected to an identical conditioning protocol, but received saline injections on all 10 conditioning days. Across conditioning, morphine-treated rats showed increased locomotor activity with subsequent injections (**Fig. 1B**), a phenomenon known as locomotor sensitization, which occurs alongside neurophysiological changes that underlie the rewarding effects of opioid drugs [44,45]. During the preference test performed two days after conditioning, morphine-treated rats showed CPP as evidenced by increased time spent in the apparatus' drug-paired side (**Fig. 1C**; **Supplementary Fig. 1**). Immediately following the preference test, animals were briefly removed from the apparatus and an aversive stimulus (cat saliva) was placed in the drug-paired side (see details in the Methods section). Cat saliva has been shown to elicit innate defensive behaviors in rodents, including avoidance of the cat odor source [29,30]. After the addition of cat odor, rats were then returned to the apparatus for a motivational conflict test in which animals had to approach cat odor to visit the drug-associated chamber. We observed that saline-treated

rats avoided the side of the apparatus containing cat odor (**Fig. 1D *left***; **Supplementary Fig. 1**). In contrast, opioid-treated rats continued to enter the drug-paired side despite the presence of cat odor, suggesting that morphine conditioning increases risk-taking behavior (**Fig. 1D *left***; **Supplementary Fig. 1**). Moreover, morphine-treated rats displayed reduced defensive responses as measured by lower levels of freezing behavior as compared to saline controls (**Fig. 1D *right***).

Because morphine-treated rats exhibited decreased freezing levels during the conflict test, we questioned if opioid exposure leads to a reduction in general defensive responses to cat odor. To test this, we performed a new experiment using the same dosing schedule as before, but instead of conditioning the animals in the apparatus, we returned them to their homecages following morphine injections (**Supplementary Fig. 2A**). During the cat odor test performed two days after the last injection, morphine- and saline-treated rats spent similar amounts of time in the side of the apparatus containing cat odor (**Supplementary Fig. 2B *left***). We observed that cat odor evoked increased freezing behavior in both saline- and morphine-treated rats compared to baseline (two-way repeated measures ANOVA, Cat Odor main effect, F (1, 18) 33.67, $p < 0.0001$, Šidák's multiple comparisons tests, saline: $p = 0.0048$, morphine: $p = 0.0004$), and the two groups exhibited similar defensive responses to cat odor as measured by either freezing (**Supplementary Fig. 2B *center***) or total distance traveled in the apparatus (**Supplementary Fig. 2B *right***) during the test. These results suggest that repeated exposure to morphine, in the dose and schedule used in our main experiment, does not affect general defensive responses to cat odor outside of a drug-associated context.

The rewarding effects of morphine are dose-dependent [46]. To test whether conditioning with a lower dose of morphine can produce risk-taking behavior in our model, we repeated the same experiments using injections of 5 mg/kg, half of our previous dose. This dose of morphine was sufficient to produce behavioral sensitization during conditioning, as shown by

increased distance traveled across days (**Supplementary Fig. 3A**). However, rats injected with this lower dose exhibited neither CPP during the preference test (**Supplementary Fig. 3B**; **Supplementary Fig. 3G**) nor risk-taking behavior during the conflict test (**Supplementary Fig. 3C**; **Supplementary Fig. 3G**). These results suggest that behavioral sensitization and CPP are dissociable and dose-dependent phenomena, and reveal that higher doses of morphine are required to elicit risk-taking behavior in rats.

Next, we sought to determine whether fentanyl, a synthetic opioid drug ~100 times more potent than morphine that increasingly contributes to overdose-related deaths in humans [47], can induce risk-taking behavior in our model in a manner similar to that observed with morphine. As with morphine, we performed experiments using two separate doses of fentanyl (0.02 or 0.04 mg/kg). Both fentanyl doses led to behavioral sensitization during conditioning (**Supplementary Fig. 3D**) and CPP during the preference test (**Supplementary Fig. 3E**; **Supplementary Fig. 3G**). However, as we have observed with morphine administration, only rats treated with the higher dose of fentanyl showed risk-taking behavior during conflict (**Supplementary Fig. 3F**; **Supplementary Fig. 3G**), reinforcing our observation that higher doses of opioids are necessary to promote risky behavior. These results also indicate that although a drug's ability to induce contextual reward memory is associated with its potential to affect risk-related decision making, these effects appear to be separable.

While sex differences in opioid addiction-related behaviors have long been reported [48, 49], broad interpretation of individual findings across specific behavioral domains has recently been called into question [50]. Additionally, research investigating sex differences in opioid seeking despite negative consequences is scarce, and the few existing results are largely inconclusive [16,51]. To investigate sex differences in risk-taking behavior after exposure to opioids, we performed the same experiments (described above) in adult female rats using the highest doses of either morphine or fentanyl that produced behavioral sensitization, CPP, and

risk-taking behavior in males. We found that female rats showed behavioral sensitization during conditioning with either morphine or fentanyl (**Supplementary Fig. 4A**). However, during the preference test, only females that were injected with fentanyl showed CPP (**Supplementary Fig. 4B *left***, **4B *right***; **Supplementary Fig. 4D**). Furthermore, fentanyl-conditioned females displayed increased risk-taking behavior during the conflict test, while those conditioned with morphine did not (**Supplementary Fig. 4C *right*, 4C *left***; **Supplementary Fig. 4D**). We conducted further experiments solely with males using the highest dose of morphine (10 mg/kg), which produced consistent behavioral effects in this group of animals.

Studies of risky drug seeking in other models identified two distinct behavioral phenotypes in drug-exposed rodents: i) those that ceased drug taking when confronted with risk, and ii) those that were aversion-resistant and continued to pursue drugs despite risk [8,9,52–54]. To determine if differential risk-taking phenotypes also exist within our model, we first identified behaviors we considered relevant to risk taking, including CPP (**Fig. 1C**), cat odor aversion (reduction in CPP from the preference test to the conflict test; **Fig. 1D *center***), and freezing (**Fig. 1D *right***). We then performed a K-means clustering of data from these three measures, which revealed two subgroups of morphine-treated rats. The first subgroup showed enhanced CPP and increased risk-taking behavior during conflict (Risk-Takers, RTs), whereas the second subgroup exhibited suppressed CPP and pronounced cat odor avoidance during conflict (Risk-Avoiders, RAs; **Fig. 2A**). Importantly, when we plotted data from saline-treated rats alongside data from RAs and RTs on these three dimensions, we observed a 40% (12 of 30 animals) overlap of the saline-treated group cluster with the RA group cluster, in stark contrast to a mere 3% (1 of 30 animals) overlap with the RT group cluster (**Supplementary Fig. 5A**; see Methods section). Compared to RAs, RTs demonstrated greater measures of CPP during both the preference and conflict tests (**Fig. 2B**; **Fig. 2C *left***; **Supplementary Fig. 5B**), as well as a lack of aversion to cat odor during the conflict test (**Fig. 2C *center***; **Fig. 2D–E; Supplementary Fig. 5B**). Compared to saline-treated

rats, RTs similarly showed CPP during the preference and conflict tests (**Supplementary Fig. 5C**; **Supplementary Fig. 5D *left***), and reduced cat odor aversion (**Supplementary Fig. 5D *center***). In contrast, RAs did not differ from saline-treated rats on measures of CPP in the preference or conflict tests (**Supplementary Fig. 5C**; **Supplementary Fig. 5D *left***), nor cat odor aversion (**Supplementary Fig. 5D *center***). Notably, while only RTs exhibited reduced freezing during the conflict test compared to saline-treated rats (**Supplementary Fig. 5D *right***), RAs and RTs did not differ in levels of freezing (**Fig 2C *right***; **Supplementary Fig. 5D *right***), further suggesting that opioid-induced risk-taking behavior is not due to a general suppression of defensive behavior.

Some of the previous studies that identified individual risk-taking phenotypes in drug-seeking animals found that risk taking was associated with other addiction-like behaviors, including increased drug intake, higher progressive ratio breakpoints, and drug-induced reinstatement of seeking responses [8,9,52,53]. To determine if risk taking in our model was similarly associated with separate addiction-like behaviors, we performed correlation analyses between behavioral measures obtained during the conflict test (paired-side preference or aversion) and those obtained earlier in the experiment (during conditioning or the preference test). We found that the expression of paired-side preference or aversion during the conflict test was not correlated with measures of behavioral sensitization during conditioning, neither when saline or morphine groups were analyzed as wholes nor when RA or RT groups were analyzed individually (**Supplementary Fig. 5E**, **Supplementary Fig. 6A–D**). RAs and RTs similarly showed no correlations between paired-side preference during the preference and conflict tests. Nonetheless, we did find that paired-side preference during the conflict test was correlated with increased CPP during the preference test in both groups of morphine-treated rats combined, but not in saline-treated controls (**Supplementary Fig. 6E–H**). This significant correlation between behaviors in the preference and conflict tests observed exclusively in the morphine-treated

group suggests that the ability of morphine to induce risk-taking behavior in our model is strongly linked to its capacity to elicit contextual reward memory at the individual level. This idea aligns with our findings from correlation analyses of female rat data in that there was no association between either behavioral sensitization and CPP (**Supplementary Fig. 7A–B**) or CPP and risk-taking behavior at a non-rewarding dose of morphine (**Supplementary Fig. 7C–D**). Taken together, these results indicate that contextual conditioning with opioids leads to the formation and expression of reward memory, as well as the emergence of risk-taking behavior during approach-avoidance conflict.

**Prelimbic cortex neurons show suppressed firing rates following acute, but not repeated, morphine administration**

To determine if opioid-induced risk-taking behavior is associated with changes in PL neuronal activity after opioid administration, we used *in vivo* single-unit electrophysiology to record PL neurons at different time points throughout the course of our behavioral experiments. Our first question was whether acute administration of morphine affects PL neuron firing rates in freely-moving rats. To answer this question, we performed recordings before and after the first morphine injection on the first day of conditioning (**Fig. 3A *left***). We used a Z-score-based method of classifying neurons as responsive given their firing rate changes pre- to post-injection (Z-score < -1.96 for inhibition, or > 2.58 for excitation). Morphine acutely suppressed PL neuron firing rates in both RAs and RTs (13% or 15% of the neurons, respectively), and these proportions of inhibited cells were significantly greater than the proportion of cells that were inhibited in saline-treated rats (5.5%; **Fig. 3B and 3D–E**). In addition, RAs showed a proportion of PL neurons that responded with increased firing rates to acute morphine injection (**Fig. 3B and 3D–E**), an effect that was not observed in RTs (**Fig. 3B and 3D–E**).

To determine if morphine's effects on PL firing rates persist across conditioning sessions, we also performed recordings before and after the final administration of morphine on the last day of drug conditioning (**Fig. 3A *right***). After the final injection, morphine failed to suppress PL firing rates in comparison to repeated saline administration (**Fig. 3C and 3F–G**). Interestingly, increases in neuronal firing rates observed in RAs during acute morphine administration were maintained during the final morphine conditioning day, differing again from RTs, which did not show increased neuronal firing (**Fig. 3C and 3F–G**). These results suggest that although morphine initially suppresses PL activity, there is adaptation to the repeated effects of morphine that renders PL neurons insensitive to this firing rate suppression by the drug. Furthermore, the persistent increase in PL firing rates in RAs following morphine administration during conditioning may underlie the failure of these animals to both form strong contextual drug reward memories and, ultimately, to exhibit risk-taking behavior during the conflict test.

**Risk-Takers show persistent inhibitory responses in PL upon initiating exploration of the paired side during conflict**

In a CPP task, rats must rely on contextual information to identify a drug-associated context while freely exploring the apparatus [24]. Previous studies have shown that PL neurons can encode reward locations in an environment, rapidly adapting as reward locations change and as animals adjust goal-directed navigational strategies to obtain these rewards [55–70]. To determine if individual PL neurons display firing rate changes upon exploration of the drug-associated context, we performed single-unit electrophysiological recordings in freely-moving rats during both the preference and conflict tests. First, we aligned neuronal spiking activity to the moment at which rats' heads entered the drug-paired side of the apparatus (**Fig. 4A**). We then applied spectral clustering, an unsupervised learning algorithm, based on these Z-score-normalized firing rates to identify groups of PL neurons with similar temporal firing patterns during

paired side entries in both tests (319 neurons from 22 rats). From this analysis emerged 7 distinct clusters of PL neurons, each with unique response profiles (**Fig. 4B–D**). We confirmed the segregation of clusters using tSNE transformation which we visualized in a 2D plot, comparing tSNE scores across each cluster pair (**Supplementary Fig. 8A–B**). One cluster in particular, Cluster 2, which included neurons with higher firing rates during paired side entries in the preference test vs. the conflict test, represented a greater ratio of neurons recorded from morphine-treated rats (RAs and RTs) than from saline-treated rats (**Fig. 4B**). This result suggests a dynamic processing of drug-associated context in PL that reflects the transition from drug seeking to approach-avoidance conflict.

To examine possible conflict-associated shifts in contextual processing by PL neurons in greater detail, we visually categorized neurons based on their individual firing rate responses, or non-responses, to paired side head entries. In all three groups of rats, we identified distinct populations of PL neurons that were either excited (Z-score > 2.58) or inhibited (Z-score < 1.96) during paired side head entries in the preference test, with lower proportions of responsive neurons in RTs than in saline-treated rats (**Fig. 4E**), suggesting that individual PL neurons display firing rate changes upon exploration of both drug-associated and neutral contexts.

After we identified PL neurons that exhibited firing rate changes associated with paired side head entries, we investigated whether these signals of exploration were altered in the presence of cat odor when rats adopted new behavioral strategies (either risk avoiding or risk taking). To answer this question, we compared the activity of paired side-responsive neurons during the preference test, described above, to their activity when aligned to paired side entries during the conflict test (**Fig. 4F–K**). In saline-treated rats, excitatory responses to paired side entries remained unchanged between the preference and conflict tests (**Fig. 4F**, **Supplementary Fig. 9A**); however, PL neurons that were inhibited during the preference test showed attenuated responses (i.e., less inhibition) to paired side entries during the conflict test (**Fig. 4I**,

**Supplementary Fig. 9B**). This finding suggests that in PL, inhibitory neuronal signals of exploration of a neutral context are suppressed once the context becomes associated with an aversive stimulus.

We next performed the same analyses in RAs and RTs to determine if the motivational conflict induced by the presence of cat odor would modify PL neuronal activity during the exploration of the drug-associated context. In contrast to saline-treated rats, excitatory paired-side entry responses were suppressed during the conflict test in both RAs (**Fig. 4G**, **Supplementary Fig. 9C**) and RTs (**Fig. 4H**, **Supplementary Fig. 9E**), suggesting that in PL, excitatory signals of morphine-associated context exploration are lost when the context acquires conflicting valences. Because inhibitory PL responses to paired side exploration were attenuated during the conflict test in saline-treated rats (**Fig. 4I**, described in the previous paragraph), we asked if the same would be true for morphine-treated rats. In RAs, inhibitory responses to paired side exploration were attenuated during the conflict test in a manner similar to that observed in saline-treated rats (**Fig. 4J**, **Supplementary Fig. 9D**). In contrast, RTs differed from both RAs and saline-treated rats in that PL neurons that were inhibited during paired side exploration in the preference test showed similar inhibitory responses during the conflict test (**Fig. 4K**, **Supplementary Fig. 9F**). This persistent inhibitory signaling of the drug-associated context from the preference to the conflict tests in RTs mirrors the persistent exploration of the drug-paired side observed during the conflict test in this subgroup of rats. Taken together, our results indicate that persistent inhibitory signaling of the drug-associated context in a subset of PL neurons during conflict may underlie increased risk taking following opioid exposure.

## DISCUSSION

We established a novel behavioral paradigm to study drug-induced risk-taking behavior by allowing rats the choice to explore either a neutral or an opioid-conditioned context, then

introducing approach-avoidance conflict by applying an aversive stimulus, cat odor, to the previously opioid-conditioned context. We found that opioid drug conditioning led to context-dependent risk-taking behavior that manifested as persistent CPP despite the coincident presence of predator threat in one subset of animals (Risk-Takers, RTs), but not in another (Risk-Avoiders, RAs). Through *in vivo* single-unit recordings, we observed that a significant proportion of PL neurons exhibited suppressed firing rates in response to acute morphine injection, but this phenomenon was not observed following repeated morphine exposure. We also observed that RAs, exclusively, exhibited increased PL firing rates upon morphine exposure, a response that persisted throughout conditioning. Single-unit recordings during the preference and conflict tests further revealed distinct populations of PL neurons that showed firing rate changes consistent with representation of environmental cues relative to the rat's location within the apparatus, with a greater number of neurons encoding the drug-paired context in RAs than in RTs or saline control rats. We also found subsets of PL neurons that displayed firing rate changes associated with rats' transitions between contexts within the apparatus. In both RA and saline groups, PL neurons that showed suppressed firing rates when rats entered the drug-paired context during the preference test lost this inhibitory response during the conflict phase in the presence of predator odor. However, this inhibitory signal of drug-paired side exploration persisted in RTs during the conflict test, mirroring the risk-taking behavior observed in this group of rats. Together, our results suggest a mechanism of opioid-induced risk-taking behavior comprised of failed prefrontal signaling of threat when drug- and threat-associated cues occur in the same context.

In our experiments, conditioning with opioid drugs (morphine or fentanyl) led to the formation of contextual reward memory and subsequent risk-taking behavior. These results resemble those from prior rodent studies using opioid self-administration punished by electrical footshocks, which reported near complete suppression of opioid seeking as a function of footshock intensity [6,7,10–15,19]. However, similar studies have reported either incomplete

suppression of opioid seeking or divergent behavioral responses that resulted in subgroups of either punishment-sensitive or punishment-resistant animals [8,9]. It is important to note that our model differs from footshock studies in that we used a non-nociceptive aversive stimulus (cat saliva) to avoid potential confounds associated with opioids' documented effects on nociceptive sensitivity [21,22]. Other rodent studies have attempted to model risky opioid seeking using non-nociceptive stimuli by employing air puffs [71], the bitterant quinine for oral self-administration [51], or co-administration of histamine [11,72] as response-contingent aversive stimuli. While these studies did not explore individual behavioral differences, none of the non-nociceptive stimulus employed were sufficient to eliminate opioid seeking overall. Thus, ours is the first study to report subgroup divergence in risky opioid seeking using a non-nociceptive stimulus as a deterrent, enabling us to further explore the neural correlates underlying distinct behavioral phenotypes during motivational conflict.

We also report sex differences in sensitivity to risk-taking behavior following comparable doses of morphine. Particularly, conditioning with 10 mg/kg of morphine led to CPP and risk-taking behavior during conflict in male rats, but this dose was insufficient to produce either of these behaviors in female rats. Previous studies have reported similar levels of opioid CPP in male and female rodents [73–76], suggesting no significant sex differences in the rewarding effects of opioids. Conversely, other studies have reported enhanced opioid CPP in female vs. male rats, indicating greater sensitivity to the rewarding properties of opioids in females [77–83]. Our findings, which demonstrate reduced CPP responses to morphine in females, agree with the few studies reporting lower opioid CPP in females compared to males [84,85]. Furthermore, we report reduced risk-taking behavior in female rats following morphine conditioning alongside reduced CPP in this group. Notably, we find no direct correlation between CPP and risk-taking behavior in females. Thus, the interplay among opioid reward, context, and risk taking is a

multifaceted factor, and our results underscore the importance of sex as a critical variable in understanding these interactions.

Opioid drugs, including morphine, have been found to suppress neuronal activity in mPFC regions [86], an effect that may contribute to the initial locomotor effects of these drugs [43,87]. In our study, we observed a reduction of PL firing rates following acute morphine administration, supporting the idea that PL is susceptible to opioid-induced alterations in neuronal activity. However, following repeated morphine administration during CPP, PL neurons underwent adaptation and no longer exhibited suppressed firing rates in response to opioid administration. Studies examining neural adaptations associated with repeated opioid exposure have revealed complex changes in PL neurons, including alterations in synaptic strength, dendritic spine morphology, glutamate receptor expression, among others [88–93]. Prior studies measuring the firing activity of PL neurons in response to acute opioid administration have resulted in contrasting findings; while some studies have reported increased excitability [42,94,95], others have shown inhibitory effects [96]. In our study, we rather explored how changes in PL firing rates in response to morphine are modified by previous repeated exposure to the drug, which has greater translational relevance for understanding behavioral adaptation during recurring opioid use. Our additional result of morphine-induced PL excitation exclusively in RAs, an effect that did not relinquish with repeated administration, further suggests that both initial and long-term neuronal responses to opioids in PL may be important in predicting subsequent risk-taking behavior. Together, our findings demonstrate that opioid-induced plasticity in PL is associated with individual differences in risk-related decision making during approach-avoidance conflict [9].

Single-unit recordings from PL neurons during our conflict test provided us a unique perspective into how risky drug seeking is processed in the brain. We found that an inhibitory neuronal signal of drug context persisted during conflict in risk-taking rats, but this signal was abolished during conflict in risk-avoiding rats. Reduced PL activity has previously been reported

in risk-taking rats during approach-avoidance conflict [97]. Recent work from our lab identified a similar pattern of inhibition in PL glutamatergic neurons of risk-taking rats during a natural reward-seeking conflict test, and optogenetic-mediated inhibition of these cells during food cue presentations in risk-avoiding rats was sufficient to restore reward seeking despite threat [39]. Further support for a role of PL in risky drug seeking comes from a separate study showing reduced activity of the PL to ventral striatum pathway in footshock-resistant rats during methamphetamine seeking [31], which is consistent with earlier reports of PL hypofunction in cocaine seeking despite punishment [41,53,98]. While we observed a subpopulation of PL neurons with persistent inhibition during risk taking in our model, we also found neurons with concurrent excitatory responses, as well as a nonresponsive majority (see **Fig. 4E**). Given the intricacy of PL dynamics, the unknown genetic identity of the neurons involved, and our model's focus on exploratory behavior rather than temporally pre-defined cue responses, we opted to forego chemo- or optogenetic manipulations that might bias PL activity across temporal and spatial scales incongruent with the single-unit activity we observed. Future studies in our lab aim to adapt our cat odor conflict model to facilitate cued drug seeking alongside appropriate neural manipulations for causal testing. In addition to PL's role in models of risky drug seeking, there may also be value in investigating the contribution of PL in other opioid-sensitive models of risk taking, especially those that involve seeking natural rewards [99,100]. Overall, our findings establish a prefrontal neural signal of risky opioid seeking in a threatening context. Our results can inform translational studies to identify prefrontal neural biomarkers in humans who might be prone to risky opioid use, as well as clinical studies that target prefrontal activity as a therapeutic approach in opioid use disorder patients.

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## AUTHOR CONTRIBUTIONS

C.Q., A.M.V., and D.S.E. performed the behavioral experiments. C.Q. and G.A.-M. performed the stereotaxic surgeries. C.Q. and A.M.V. performed the single-unit recording experiments. C.Q., E.P.B, C.L.C., X.O.Z., and F.H.D.-M. analyzed the single-unit recording experiments. All the authors participated in the design of the experimental protocols and interpretation of the data. C.Q. and F.H.D.-M. prepared the manuscript with the comments from all the co-authors.

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## COMPETING INTERESTS

The authors declare no competing interests.

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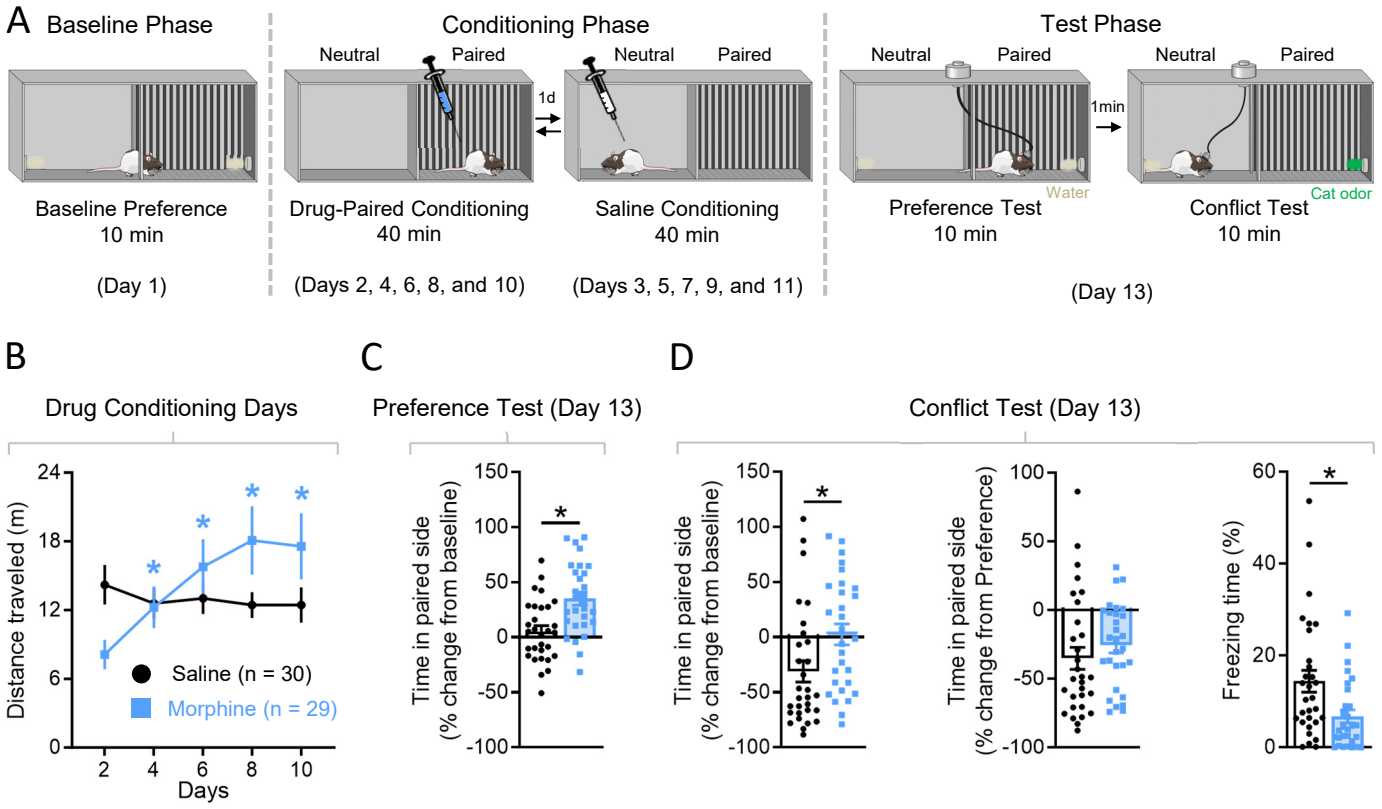


**Figure 1. Repeated morphine administration leads to contextual reward memory formation and risk-taking behavior during approach-avoidance conflict.**

**A)** Schematic timeline of morphine conditioned place preference and approach-avoidance conflict tests. **B)** Distance traveled in the 10-min period following acute drug administration over the 5 days of drug conditioning. Morphine-treated rats showed increased locomotor activity beginning on day 4 of conditioning; however, saline-treated rats did not (two-way repeated measures ANOVA, Day X Drug interaction, $F(4, 228) = 13.18$, $p < 0.0001$, Šidák's multiple comparisons tests); asterisks denote difference from baseline within the Morphine group, $^*p < 0.05$). **C)** Percentage of change from baseline in time spent in the drug-paired side of the apparatus. Morphine-treated rats exhibited conditioned place preference as measured by the increased amount of time in the drug-paired side compared to saline controls (Unpaired Welch's t-test, $^*p < 0.001$). **D *left*)** Percentage of change from baseline in time spent in the drug/cat-paired side of the apparatus during the conflict test. Rats conditioned with saline, but not morphine, showed aversion to the drug/cat-paired side (Shapiro-Wilk normality test, $p < 0.001$; Unpaired Mann-Whitney *U*-test, $^*p = 0.0062$). **D *center*)** Percentage of change from the preference test in time spent in the drug/cat-paired side of the apparatus during the conflict test. No groups differences were observed (Shapiro-Wilk normality test, $p = 0.007$; Unpaired Mann-Whitney *U*-test, $p = 0.149$). **D *right*)** Percentage of time spent freezing during the conflict test. Morphine-treated rats displayed reduced freezing levels compared to saline controls (Shapiro-Wilk normality test, $p < 0.05$; Unpaired Mann-Whitney *U*-test, $^*p = 0.0053$). Data are shown as mean ± SEM.

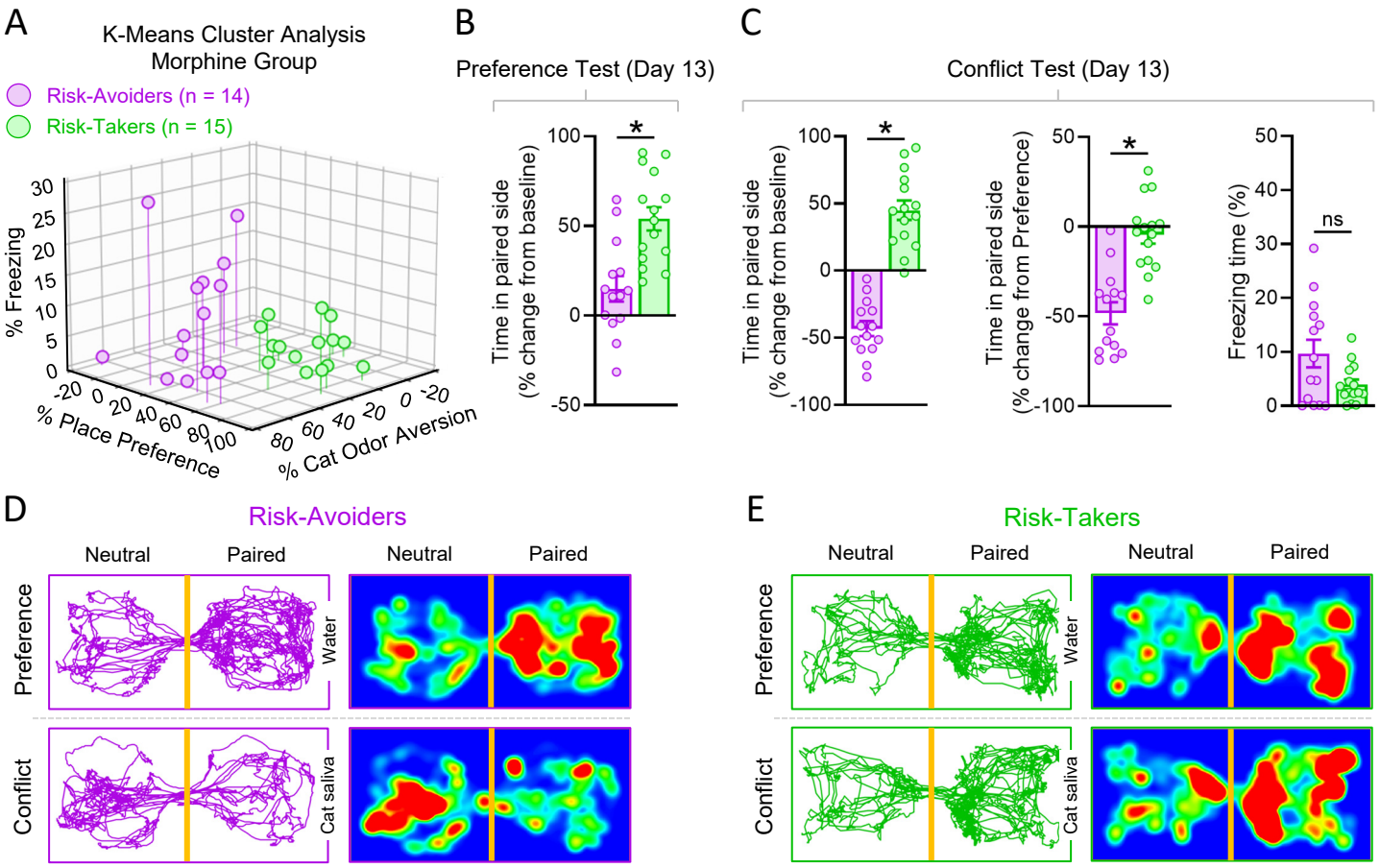


**Figure 2. Morphine-treated rats show individual differences in risk-taking behavior during conflict.**

**A)** K means cluster analysis (10 repetitions) of Morphine group animals in measures of freezing (% time spent freezing during the conflict test), place preference (% change from baseline in time spent in the drug-paired side during the preference test), and cat odor aversion (% change from preference test in time spent in the drug/cat-paired side during the conflict test). Two clusters were identified: one with lower place preference and greater cat odor aversion (magenta cluster, Risk-Avoiders, n = 15), and another with greater place preference and lower cat odor aversion (green cluster, Risk-Takers, n = 14). **B)** Percentage of change from baseline in time spent in the drug-paired side of the apparatus. Risk-Takers demonstrated greater place preference than Risk-Avoiders (Unpaired Welch's t-test, *$p < 0.001$). **C *left*)** Percentage of change from baseline in time spent in the drug/cat-paired side of the apparatus during the conflict test. Risk-Takers showed less cat odor aversion than Risk-Avoiders (Unpaired Welch's t-test, *$p < 0.0001$). **C *center*)** Percentage of change from the preference test in time spent in the drug/cat-paired side of the apparatus during the conflict test. Risk-Takers showed less cat odor aversion than Risk-Avoiders (Unpaired Welch's t-test, *$p < 0.0001$). **C *right*)** Percentage of time spent freezing during the conflict test. Both groups displayed similar levels of freezing during the conflict test (Unpaired Welch's t-test, *$p = 0.052$). Representative tracks and heatmaps of time spent in either side of the apparatus during Preference or Conflict Tests for Risk-Avoiders **(D)** and Risk-Takers **(E)**. Data are shown as mean ± SEM.

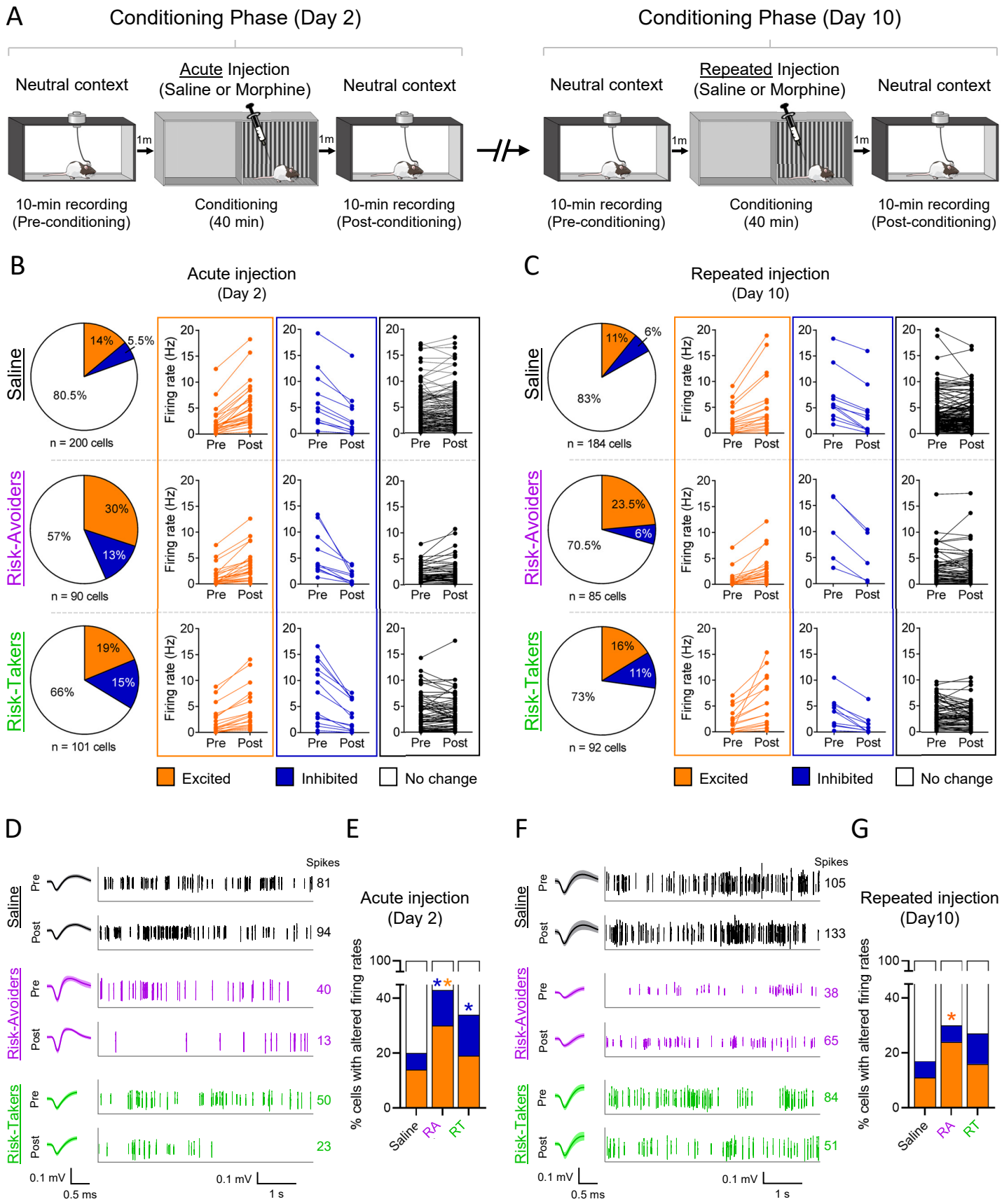


**Figure 3. Morphine-induced PL inhibition is lost after conditioning in both Risk-Avoiders and Risk-Takers, but persistent PL excitation is exclusive to Risk-Avoiders.**

**A)** Timeline of recordings of spontaneous PL single unit activity after acute (Day 2) and repeated (Day 10) administration of saline or morphine (10 mg/kg, subcutaneous). **B)** Representations of cells excited (orange),

inhibited (dark blue), or exhibiting no change (black/white) in response to acute drug administration (z-scores used for response classification [excited, 2.58; inhibited, -1.96]; cells with firing rates >20 Hz not shown [Saline = 7, Risk-Avoiders = 3, Risk-Takers = 3]). As compared to saline administration, acute morphine administration resulted in a greater number of cells showing increased firing rates in Risk-Avoiders (Fisher exact test, ratio of cells excited to not excited, $p = 0.002$), and decreased firing rates in both Risk-Avoiders and Risk-Takers (Fisher exact tests, ratio of cells inhibited to not inhibited; Risk-Avoiders: $p = 0.0327$; Risk-Takers: $p = 0.0089$). **C)** Representations of cells excited (orange), inhibited (dark blue), or exhibiting no change (black/white) in response to acute drug administration (z-scores used for response classification [excited, 2.58; inhibited, -1.96]; cells with firing rates >20 Hz not shown [Saline = 3, Risk-Takers = 1]). On the final drug administration day, morphine failed to suppress PL cell firing rates beyond what was observed after saline administration (Fisher exact tests, ratio of cells inhibited to not inhibited; Risk-Avoiders: $p = 1.000$; Risk-Takers: $p = 0.156$). However, in Risk-Avoiders, increased PL cell firing rates in response morphine administration were maintained relative to animals that were administered saline (Fisher exact test, ratio of cells excited to not excited, $p = 0.0091$) **D)** Example waveforms and spike raster plots (5s samples from 300s to 305s during recordings) of two representative cells, one from either group, during baseline and after saline or morphine administration on Day 2 (no change cell from Saline group; inhibited cells from Risk-Avoider and Risk-Taker groups). Numbers to the right of spike raster plots denote the quantity of spikes shown. **E)** Relative percentages of cells on Day 2 that were excited (Fisher exact tests, RAs vs. saline: $p = 0.033$, RTs vs. saline: $p = 0.009$, RAs vs. RTs: $p = 0.84$), inhibited (Fisher exact test, RAs vs. saline: $p = 0.002$, RTs vs. saline: $p = 0.31$, RAs vs RTs: $p = 0.09$), or exhibited no change in response to saline or morphine administration (*$p < 0.05$). **F)** Example waveforms and spike raster plots (5s samples from 300s to 305s during recordings) of two representative cells, one from either group, during baseline and after saline or morphine administration on Day 10 (no change cells from Saline and Risk-Taker groups; excited cell from Risk-Avoider group). Numbers to the right of spike raster plots denote the quantity of spikes shown. **G)** Relative percentages of cells on Day 10 that were excited, inhibited, or exhibited no change in response to saline or morphine administration. Injections failed to result in significant inhibition (Fisher exact tests, RAs vs. saline: $p = 1.0$, RTs vs. saline: $p = 0.16$, RAs vs. RTs: $p = 0.29$), but continued to result in significant excitation solely in the Risk-Avoider group (Fisher exact tests, RAs vs. saline: $p = 0.009$, RTs vs. saline: $p = 0.25$, *$p = 0.0091$).

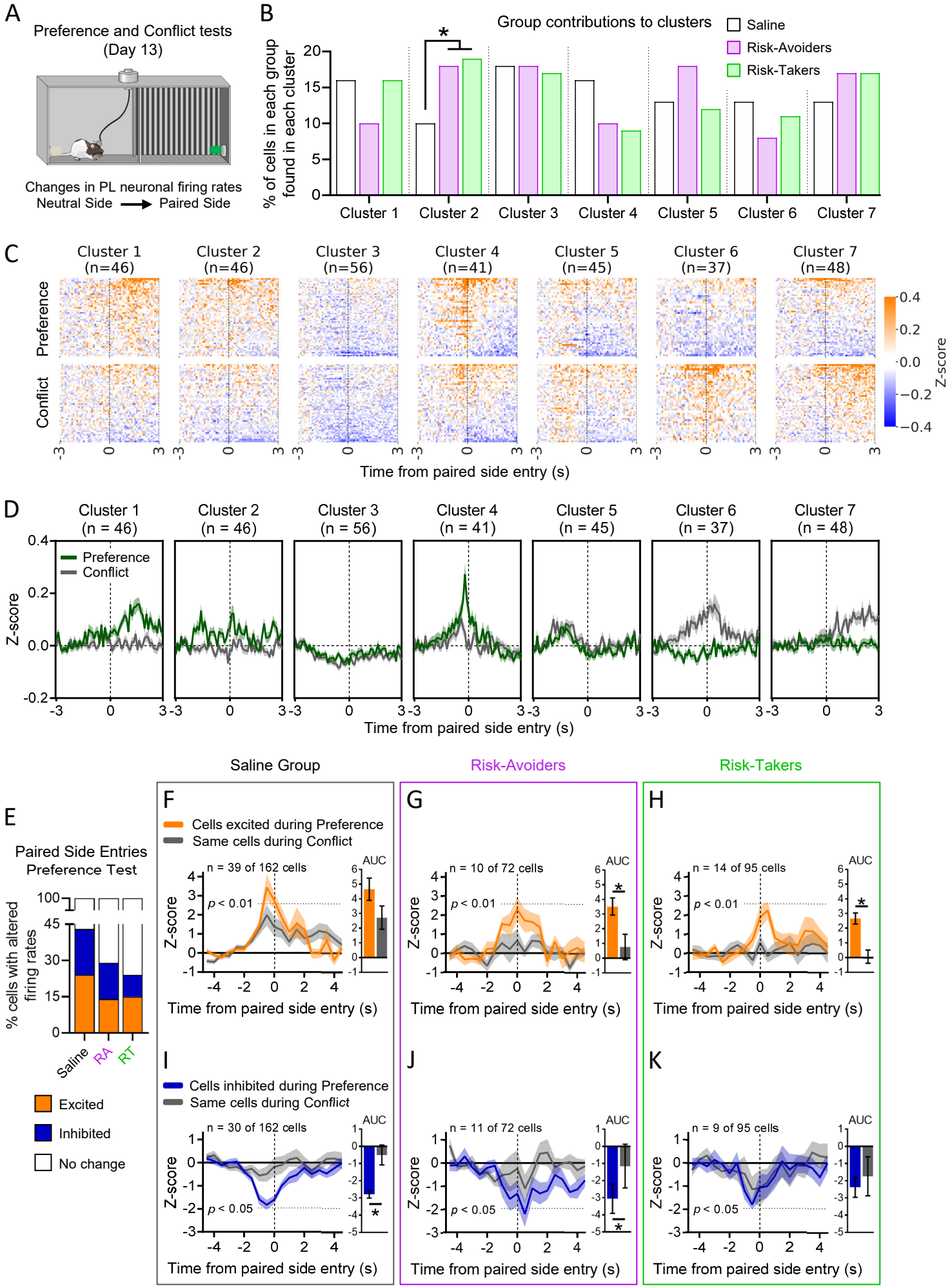


**Figure 4. Risk-Takers show persistent inhibitory responses in PL upon initiating exploration of the paired side during conflict.**

**A**) Schematic diagram demonstrating the behavior (paired side head entries) to which neuronal activity was aligned for the following analyses. **B**) Percentages of cells identified from recordings from each group that were included in Clusters 1 through 7 after spectral clustering. A larger proportion of PL cells in morphine-treated rats (Risk-Avoiders and Risk-Takers) exhibited paired side entry responses consistent with Cluster 2 compared to Saline-treated rats (Fisher exact test, saline vs. morphine: $p = 0.038$). **C**) Single-unit peri-event raster plots and **D**) mean peri-event time histograms showing firing rate changes of PL cells relative to paired-side entries in the preference and conflict tests. All data shown as Z-scores. **E**) Percentages of cells showing excitation (z-score > 2.58), inhibition (z-score < -1.96), or no response to paired side entries during the preference test (saline-treated group, n = 9 rats: 39/162 [24%] cells excited, 30/162 [19%] cells inhibited; RA group, n = 6 rats: 10/72 [14%] cells excited, 11/72 [15%] cells inhibited; RT group, n = 7 rats: 14/95 [15%] cells excited, 9/95 [9%] cells inhibited; Fisher exact tests, RAs vs. saline: $p = 0.059$, RTs vs. saline: $p = 0.0031$, RAs vs. RTs: $p = 0.48$). **F,G,H**) Graphs representing firing rate changes of PL cells in Saline (**F**; n = 9 rats, AUC, $p = 0.070$), Risk-Avoider (**G**; n = 6 rats, AUC, $p = 0.02$), and Risk-Taker (**H**; n = 7 rats, AUC, $p = 0.0013$) groups that showed excitatory spontaneous activity (z-score > 2.58) when animals crossed into the paired side during the preference test (orange) compared to firing rates of the same cells when animals crossed into the paired side during the conflict test (charcoal). Inset bar graphs show differences in the total areas under the curves between test stages 500 msec before and after line crossings ($^*p < 0.05$). In Saline-treated rats, excited paired side entry-responsive PL cells showed similar spatial firing rate changes during both the preference and conflict tests (AUC, $p = 0.070$). However, PL neurons that responded to paired side entries during the preference test with increased firing rates did not respond to paired side entries during the conflict test in Risk-Avoiders (AUC, $p = 0.022$) or Risk-Takers (AUC, $p = 0.001$). **I,J,K**) Graphs representing firing rate changes of PL cells in Saline (**I**; n = 9 rats, AUC, $p < 0.001$), Risk-Avoider (**J**; n = 6 rats, AUC, $p = 0.022$), and Risk-Taker (**K**; n = 7 rats, AUC, $p = 0.499$) groups that showed inhibitory spontaneous activity (z-score < -1.96) when animals crossed into the paired side during the preference test (dark blue) compared to firing rates of the same cells when animals crossed into the paired side during the conflict test (charcoal). Inset bar graphs show differences in the total areas under the curves between test stages 500 msec before and after line crossings ($^*p < 0.05$). PL neurons that responded to paired side entries during the preference test with decreased firing rates did not respond to paired side entries during the conflict test in either Saline-treated rats (AUC, $p = 0.001$) or Risk-Avoiders (AUC, $p = 0.016$). However, in Risk-Takers, inhibited paired side entry-responsive PL cells showed similar spatial firing rate changes during both the preference and conflict tests (AUC, $p = 0.499$). Data are shown as mean ± SEM.