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The behavioral and neural architecture of observational active avoidance is shaped by demonstrator experience and observer sex

Shannon Ruble, Cassandra Kramer, Lexe West, Helen Durrett & Maria M. Diehl✉

Observational learning allows an individual to gain information about potential outcomes and is particularly advantageous when learning to actively avoid danger without risking harm to oneself. To assess observational learning of active avoidance, Observer rats witnessed Demonstrator rats undergo platform-mediated avoidance (PMA), in which rats learn to avoid a tone-signaled footshock by stepping onto a safe platform. We found that Observers' avoidance was correlated with a Novice, but not an Experienced, Demonstrator, suggesting that Novice and Experienced Demonstrators transmit different information to the Observer that can be used to acquire active avoidance. Shock pre-exposure enhanced avoidance in male Observers and shifted their behavioral strategy toward avoidance-preferring rather than reward-preferring responses, an effect not observed in females. We then used c-Fos immunohistochemistry to quantify neural activation in brain areas previously implicated in direct learning of active avoidance. Observers that witnessed Experienced Demonstrators showed more correlations in activation between brain areas than Observers that witnessed a Novice Demonstrator. Furthermore, these activation profiles scaled with behavioral strategy: avoidance-preferring Observers showed the greatest number of correlations, balanced Observers showed fewer, and reward-preferring Observers showed the least number of correlations. Taken together, these results provide insights into the behavioral mechanisms that promote observational avoidance learning.

Keywords Fear, Medial prefrontal cortex, Nucleus accumbens, Amygdala, Social learning, c-Fos immunohistochemistry

Observational learning allows individuals to learn about potential outcomes without directly experiencing those outcomes, thus reducing the risk of harm or missed opportunities. While observational learning has been studied for over a century[1], little is known concerning how individuals learn to avoid threats through observation. Early research on observational learning of avoidance used hurdle jumping and shuttle avoidance tasks to investigate the behavioral mechanisms promoting observational avoidance[2–4]. These studies found that subjects performed more avoidance responses following observation compared to subjects that learned through direct experience alone. However, these studies manipulated different variables such as exposing Observers to a footshock prior to training[4] or manipulating the Demonstrator's experience with the task prior to being observed[2]. Because previous studies have not manipulated both exposure to footshock and Demonstrator experience in a single experiment, it is difficult to determine which behavioral conditions best promote observational avoidance learning. Furthermore, the previous studies did not investigate the neural mechanisms of observational avoidance, leaving much about this process unknown.

Although we do not yet know the neural mechanisms of *observational* avoidance, the neural mechanisms of *direct* avoidance have been well defined. In the platform-mediated avoidance (PMA) task, the prelimbic cortex (PL) bidirectionally mediates avoidance via projections to the nucleus accumbens (NAc) and to the basolateral amygdala (BLA), whose activity decreases or increases avoidance, respectively[5]. Avoidance circuitry, however, is not the same for all active avoidance tasks. Whereas projections from the BLA to the NAc are necessary for

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avoidance expression in both PMA[5] and shuttle avoidance[6], the role of this projection is unknown in lever-press avoidance. The infralimbic cortex (IL), however, is important for the expression of avoidance in both shuttle[7–9] and lever-press avoidance tasks[10,11] but not the PMA task[12]. Furthermore, recent work has shown that the context of avoidance acquisition influences the neural mechanisms involved as the anterior cingulate cortex (ACC) plays a role in the expression of avoidance during PMA that is acquired within a social, but not a solitary, context[13]. The ACC also serves a well-established role in observational learning[14–16]. Although these subregions of the medial prefrontal cortex are not recruited for the same avoidance tasks, they do share multiple projection sites including the BLA[17–19] and the NAc[20,21].

In this study, we developed a new task that allows rats to learn the PMA task through observation of a conspecific. We determined which behavioral conditions promote optimal acquisition of observational avoidance, including whether prior shock experience is necessary, and whether observing a Novice Demonstrator (performing Day 1 of PMA training) versus an Experienced Demonstrator (performing Day 10 of PMA training) differentially affects avoidance learning. We hypothesized that rats witnessing an Experienced Demonstrator would acquire avoidance more readily than rats observing a Novice Demonstrator. We also hypothesized that shock pre-exposure would lead to increased levels of avoidance when Observers witnessed either type of Demonstrator given that prior observational fear learning research has shown that shock pre-exposure enhances learning[22–25]. We then used an immunohistochemical approach to characterize neural activation across brain regions that are known to be recruited during active avoidance in the PMA task as well as during observational learning. We hypothesized that areas necessary for avoidance expression (PL, NAcC, and BLA) would show increased activation in Observers when they witnessed an Experienced versus a Novice Demonstrator performing the PMA task given that these areas are involved in regulating avoidance expression following multiple days of training[5,12]. We also identified activation profiles for each Observer group and behavioral clusters based on correlations in activation between areas. We hypothesized that behavioral clusters would also be characterized by unique activation profiles.

Materials and methods

Subjects

A total of 208 adult Sprague-Dawley rats (116 males and 92 females) were used in this study. All experimental rats were bred in-house from breeder rats purchased from Charles River Laboratories (Wilmington, MA). Rats were housed in same-sex dyads or triads in standard cages and kept on a reverse light-dark cycle (lights off at 0830). Rats were handled at least twice weekly until females weighed 215 g and males weighed 300 g (2–3 months of age) at which point rats entered experiments and were handled daily. Rats were food-restricted to 16–18 g of standard laboratory rat chow per day to maintain at least 85% of their free-feeding weight while being trained to lever-press for sucrose pellets[26]. All procedures described here were approved by the Institutional Animal Care and Use Committee of Kansas State University in compliance with the National Institutes of Health guidelines for the care and use of laboratory animals. This study was reported in accordance with the ARRIVE guidelines.

Experimental design

In Observational PMA, an Observer rat witnessed a Demonstrator rat undergo PMA training as previously described[12,13,26]. Briefly, Demonstrators lever-pressed for sucrose reward (BioServ, Flemington, NJ) on a 30-second variable interval schedule of reinforcement (VI-30) and experienced 9 presentations of a 30-second tone that co-terminated with a 2-second footshock (4 kHz tone, 0.4 mA shock) with an average inter-trial interval (ITI) of 3 min and underwent 10 training sessions (1 session per day). The Demonstrator could avoid the tone-signaled footshocks by stepping onto a safe platform located in the corner opposite the lever. While the Demonstrator performed PMA, a same-sex Observer from a different homecage witnessed an entire training session (9 tone-shock pairings) through a transparent acrylic barrier from an adjacent chamber (Fig. 1A, **left**). The barrier was perforated, allowing the Observer to receive sensory information from the Demonstrator's chamber without interfering with the Demonstrator's performance of the task. The Observer did not have access to a lever, food dish, or platform while watching the Demonstrator to limit distractions and encourage the Observer to focus their attention on the behavior of the Demonstrator. The side of the chamber in which the Demonstrator performed PMA (left or right) was counterbalanced across pairs to control for any potential effect the side of the chamber may have had on behavior.

Demonstrators underwent PMA training for 10 days (Fig. 1B). Across the 10 days of Demonstrator PMA training, 2 different groups of Observers witnessed either a Novice Demonstrator (undergoing Day 1 of PMA training) or an Experienced Demonstrator (undergoing Day 10 of PMA training; see Fig. 1). Prior to the Observation Session, a subset of Observers was pre-exposed to a footshock. Shock pre-exposure sessions began with a 5-minute habituation period on the side of the chamber in which the Demonstrator would undergo PMA, followed by a single unsignaled footshock (0.4 mA, 2 s). Shock pre-exposed rats were left in the chamber for an additional minute following the footshock and then placed on the observer side of the chamber and underwent their Observation Session. The platform was present during the shock exposure session and sucrose was available contingent upon lever-pressing on a VI-30 schedule. Shock-naïve Observers remained in the homecage until the beginning of the Observation Session. Rats were housed with cagemates of the same experimental group (Early versus Late PMA, shock pre-exposed versus shock-naïve) to limit social transfer of information in the homecage between rats experiencing different experimental conditions. Including both shock pre-exposed and shock-naïve Observers in this study allowed us to determine whether experience with footshock enhances observational learning as has previously been seen in observational fear learning[22–25].

Twenty-four hours after observation, the Observers were placed into the chamber previously occupied by the Demonstrator and given a full PMA Test Session in which they experienced 9 tone-shock presentations (Fig. 1A, **right**). Observers that were used for c-Fos immunohistochemistry were sacrificed 90 min after Tone 1 of the Test

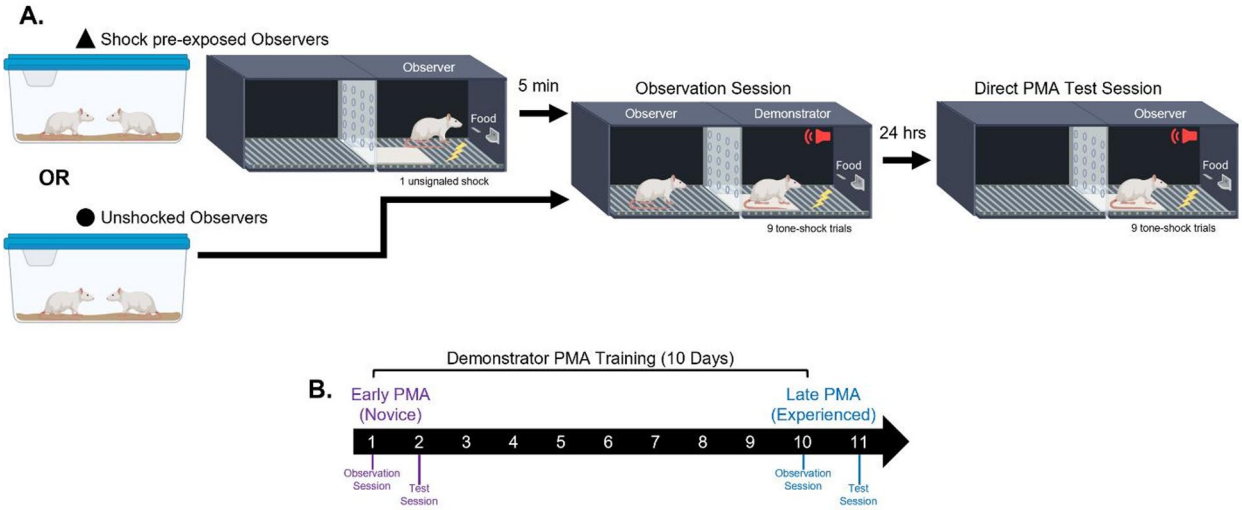

Fig. 1. Experimental design. (**A**) Observer rats are first assigned to a shock pre-exposed or shock-naïve group (left). Shock pre-exposed rats are placed on the side in which the Demonstrator will undergo PMA training and receive 1 unsignaled footshock. During the Observation Session, the Demonstrator receives 9 tone-shock trials. The Observer does not have access to a platform, lever, or food dish, but can gain sensory information from the Demonstrator through a perforated transparent barrier (middle). 24 h later, the Observer is placed in the side of the chamber that the Demonstrator previously occupied and receives 9 tone-shock pairings (right). The Observer then undergoes a Direct PMA Test Session 24 h after witnessing the Demonstrator. Created in BioRender. https://BioRender.com/xwofx4e. (**B**) Demonstrators are trained in PMA across 10 days. Observers undergo an Observation Session, in which they witness either a Novice Demonstrator undergoing Early PMA (Day 1 of PMA training, purple) or an Experienced Demonstrator undergoing Late PMA (Day 10 of PMA training, blue).

Session. This timepoint was chosen to capture the neural activation profile when Observers were required to recall the information learned through observation to perform avoidance themselves.

Behavioral data

Behavioral measures of time on platform, freezing, lever-pressing, and head orientation toward the Demonstrator were automatically scored using ANY-Maze (v7.51, www.any-maze.com) behavioral tracking software. Time on platform and freezing were automatically scored during the tone period (30s) of the Observer Test Session. The number of shocks avoided was calculated by identifying instances where the rat spent at least 1.75s of the 2s shock period on the platform. The number of lever-presses during the inter-trial interval (ITI) was measured during the 60s pretone period . The Observer was classified as oriented towards the Demonstrator when the barrier was within a 90-degree visual angle from the tip of the rat's nose (i.e., 45 degrees to either side of the nose). This range of head orientation was used because rats have a nearly 200-degree field of view, but only approximately 40 to 110 degrees of binocular vision[27,28]. Head orientation was measured during the tone period (30s) of the Observation Session when critical task information is conveyed by the Demonstrator.

Tissue collection and immunohistochemistry

A subset of animals from each experimental group, as well as a group of homecage controls, were sacrificed for c-Fos immunohistochemistry (IHC). Ninety minutes following the presentation of Tone 1 of the Observer Test Session, Observers were deeply anesthetized with sodium pentobarbital (450 mg/kg i.p.) and transcardially perfused with 0.9% saline until all blood was cleared, followed by 4% paraformaldehyde. c-Fos is an immediate early gene commonly used as a marker for neuronal activation[29,30]. It is well established that protein expression peaks between 60 and 120 min following stimulation[31], so the 90-minute time point was chosen to capture this peak expression[32–35]. Brains were removed from the skull and stored in 4% paraformaldehyde at 4 °C for 12 to 24 h. Following fixation, brains were transferred to a 20% sucrose solution in 1X PBS for 24 h followed by a 30% sucrose solution in PBS for another 48 to 72 h. Samples were then gradually cooled to -20 °C and sliced on the coronal plane (40 µm) on a cryostat and stored in cryoprotectant at -20 °C until the immunohistochemical assay was conducted.

Prior to staining, the tissue was washed in PBS (3 × 10 min) to remove cryoprotectant, then blocked in a buffer containing 10% normal goat serum, 5% bovine serum albumin, and 0.5% triton-X in PBS at room temperature (RT) for 1 h. Sections were subsequently incubated in *c-Fos* rabbit monoclonal primary antibody (1:3200, Cell Signaling, Danvers, MA) at RT for 48 h and then washed in PBS (3 × 10 min). The tissue was then incubated in a secondary anti-rabbit antibody (1:500 AlexaFluor 588, Cell Signaling, Danvers, MA) at RT for 6 h followed by PBS washes (5 × 10 min) and stained with DAPI (10 min). Finally, the tissue was washed twice with PBS (1 min and 10 min) and stored in fresh PBS overnight. Sections were then mounted on slides and coverslipped with Fluoromount-G mounting medium (Southern Biotech, Birmingham, AL).

Tissue imaging and c-Fos quantification

Following IHC, coronal sections were imaged using an Olympus BX63F fluorescence microscope and digital camera at 10x magnification. Individual Z-stack images were stitched together using cellSens Dimension (v4.4.1, evidentscientific.com) software to create an X-Y composite image across the prefrontal cortex. Z-stacks were merged to create a single plane in which all c-Fos positive cells in the 40 µm thick section could be seen simultaneously. Quantification of c-Fos positive cells was conducted automatically using QuPath[36] (v0.7.0, https://qupath.github.io). The areas of interest were delineated to calculate the total area of the region for each rat. Density of c-Fos positive cells was calculated by dividing the number of c-Fos positive cells by the total area of the brain region. c-Fos density was measured in both hemispheres from two to three non-consecutive slices and averaged to obtain a final c-Fos density for each rat[33]. Thresholding for counting was performed with the experimenter blinded to condition.

Statistical analyses

Behavioral measures of time spent freezing and time on platform were analyzed from the Observers' Test Session using multilevel beta regressions. Head orientation was analyzed from the Observers' Observation Session using a multilevel beta regression. Beta regressions were used because behaviors were measured as the proportion of each Tone spent performing that behavior, producing data bound between zero and one. Tone, condition (observed Novice or Experienced Demonstrator), shock pre-exposure status, sex, and all their interactions were specified as fixed effects in the regression models and subject ID was specified as a random effect. Planned contrasts were performed on the interaction of shock pre-exposure status and condition given that we hypothesized that shock pre-exposure, observing an experienced demonstrator, or a combination of the two would improve observational avoidance acquisition. A Sidak correction was used to maintain family wise error rate, and the pairwise comparisons were as follows: shock-naïve Observers, shock pre-exposed Observers, Observers witnessing Novice Demonstrators, Observers witnessing Experienced Demonstrators.

A Poisson regression was used to analyze the number of shocks avoided with the same fixed effect structure used for the beta regressions. A multilevel Poisson regression was used to analyze the number of lever presses made during the intertrial interval (ITI) of the Test Session with the same fixed and random effect structure used for the beta regressions. Planned contrasts for both Poisson regressions followed the same structure as those used for the beta regressions.

Correlations were conducted between Demonstrator behavior during the Observation Session and Observer behavior during the Observer Test Session. This analysis was done to assess the relationship between the behaviors the Observer witnesses and the behaviors they later perform in their Test Session. Behavioral measures were averaged across the nine tones to obtain a single data point for each subject. Additionally, correlations were conducted between the head orientation of Observers during the Observation Session and their time on platform or freezing during the Test Session.

A hierarchical cluster analysis was used to identify distinct behavioral phenotypes within Observational PMA. The variables used to define the clusters were time on platform, freezing, lever-presses during the 60s of the ITI immediately preceding the tone, and number of shocks avoided during the Observer Test Session. Time on platform, freezing, and pretone lever presses were averaged across the 9 tones of the Test Session to obtain a single value for each Observer. All variables were standardized to ensure equal weighting when defining the clusters. Euclidian distance was used to measure the dissimilarity between data points. The number of behavioral clusters was then determined from the resulting dendrogram. Fisher's exact tests were then used to determine whether the clusters were independent from the Observers' experience with footshock. We were unable to determine whether the interaction of shock pre-exposure and condition was independent from the behavioral cluster Observers fell into due to small samples from one of the clusters. We then used ANOVAs with Tukey's corrected post-hocs to compare behavioral measures across each cluster.

ANOVAs with Sidak's corrected planned contrasts were used to analyze the effect of shock pre-exposure and condition on c-Fos density. Separate ANOVAs were used for each brain area (PL, IL, ACC, NAcC, NAcS, BLA). We also conducted correlations of c-Fos density between the six brain areas measured in each Observer group. Separate ANOVAs (with Sidak's corrected post-hocs) were used to analyze differences in c-Fos density between behavioral clusters. c-Fos density was then correlated between the brain areas for each behavioral cluster.

All statistical analyses were conducted in R (v4.4.2) using the glmmTMB[37,38], corr[39], and emmeans[40] packages. JMP Pro (v19.0, www.jmp.com) was used to create correlation matrices, and GraphPad Prism (v10.0.0; www.graphpad.com) was used to create violin plots. Operant box graphics were created using BioRender (www.biorender.com).

Results

Shock pre-exposure enhances observational avoidance when Observers witness a Novice, but not an Experienced, Demonstrator

PMA requires multiple days of training in which rats transition from freezing and escape responses during early stages of PMA to avoidance of the tone CS by stepping onto the platform during late stages of PMA[12,13,41,42]. Due to the transition in behavioral responses over time, we were interested in whether Observers would acquire the task differently when they observed a Novice Demonstrator during early stages of PMA training (Day 1, when freezing and escape responses are high and avoidance is low) versus observing an Experienced Demonstrator during late stages of PMA training (Day 10, when avoidance is high and freezing and escape responses are low). In addition, prior work examining observational learning of aversive tasks, like observational fear conditioning, has found that pre-exposure to the footshock unconditioned stimulus (US) improves observational learning[22–25]. To examine how both shock pre-exposure and experience level of the Demonstrator influence Observational PMA, we used a two-by-two design, producing four separate Observer groups: shock pre-exposed and shock-naïve

Observers witnessing a Novice Demonstrator (light purple and dark purple respectively) and shock pre-exposed and shock-naïve Observers witnessing an Experienced Demonstrator (light blue and dark blue, respectively, refer to Fig. 1).

To assess avoidance learning across our different observer groups, we compared the percentage of time spent on the platform during each tone of the Test Session using a multilevel beta regression. Shock pre-exposed Observers spent significantly more time on the platform than shock-naïve Observers (z=-3.668, $p<0.001$). The interaction between shock pre-exposure and experience of the Demonstrator trended toward significance (z=-1.660, $p=0.097$). Sidak's planned contrasts revealed that when observing a Novice Demonstrator, shock pre-exposed Observers spent a significantly higher percentage of time on the platform during the tone than shock-naïve Observers (Fig. 2A; z=-3.772, $p<0.001$). Shock pre-exposure did not, however, alter the percentage of time spent on the platform when Observers witnessed an Experienced Demonstrator (Fig. 2D; z=-1.425, $p=0.488$). There were no significant differences in time on platform between Observers who witnessed Novice or Experienced Demonstrators (z = 0.179, $p=0.858$) in shock pre-exposed (Fig. 2A **vs. D**, light triangles; $z=1.249$, $p=0.614$) or shock-naïve Observers (Fig. 2A **vs. D**, dark circles; z=-1.095, $p=0.721$).

We then analyzed the number of shocks avoided at Test using a Poisson regression. We found that shock pre-exposure led to an increased number of shocks avoided (z=-4.354, $p<0.001$). Sidak corrected contrasts confirmed that this was the case when Observers witnessed either Novice (Fig. 2B; z=-3.632, $p=0.001$) or Experienced Demonstrators (Fig. 2E; z=-2.531, $p=0.045$). There were no differences in number of shocks avoided between Observers who witnessed Novice or Experienced Demonstrators ($z=0.936$, $p=0.350$) in either shock pre-exposed (Fig. 2B **vs. E**, light colors; z = 1.296, $p=0.580$) or shock-naïve conditions (Fig. 2B **vs. E**, dark colors; z = 0.116 $p=0.999$).

To assess differences in freezing and reward seeking across observer groups, we analyzed the percentage of time spent freezing during each tone using a multilevel beta regression and the number of ITI lever presses

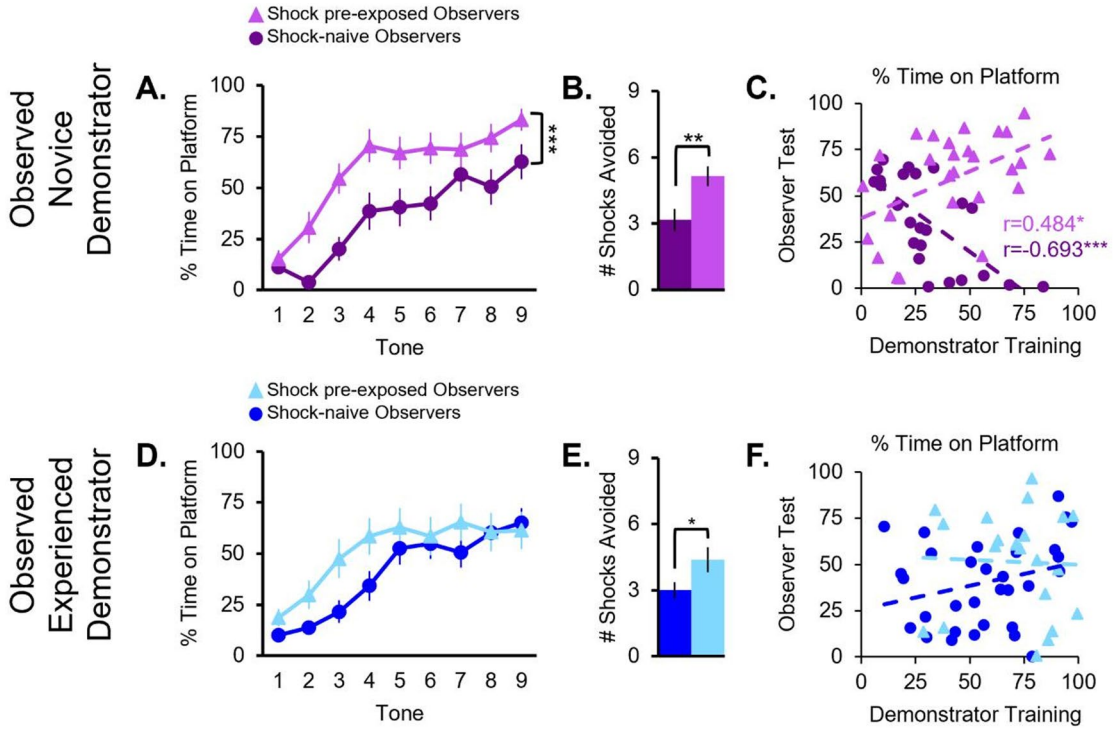

Fig. 2. Shock pre-exposure enhances avoidance learning after observing Novice, but not Experienced, Demonstrators. (**A**) Shock pre-exposure increased Observer time on platform ($z=-3.772$, $p<0.001$) and (**B**) number of shocks avoided ($z=-3.632$, $p=0.001$) after observing a Novice Demonstrator. (**C**) After witnessing a Novice Demonstrator, Observers' time spent on the platform at Test was positively correlated with the Demonstrator's time on platform for shock pre-exposed Observers (light triangles; r = 0.484, $p=0.011$) and negatively correlated for shock-naïve Observers (dark circles; r = − 0.693, $p<0.001$). (**D**) Shock pre-exposure did not alter Observer time on platform (z = − 1.425, $p=0.488$), but increased (**E**) number of shocks avoided ($z=-2.531$, $p=0.045$) after observing an Experienced Demonstrator. (**F**) After witnessing an Experienced Demonstrator, there were no significant correlations between Observers' Test Session time on platform and their Demonstrator's training time on platform in shock pre-exposed (light triangles; $r=-0.040$, $p=0.865$) or shock-naïve Observers (dark circles; $r=0.272$, $p=0.133$). $n=27$ shock pre-exposed Observers witnessing a Novice (light purple), $n=24$ shock-naïve Observers witnessing a Novice (dark purple), $n=21$ shock pre-exposed Observers witnessing an Experienced Demonstrator (light blue), $n=32$ shock-naïve Observers witnessing an Experienced Demonstrator (dark blue). Data shown as mean +/− SEM. $^{\#}p<0.10$, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

using a multilevel Poisson regression. There were no significant differences in freezing or lever-pressing between shock pre-exposed and shock-naïve Observers witnessing Novice (**Figure S1A**; z=-2.313, $p = 0.080$; **Figure S1C**; z = 2.384, $p = 0.067$) or Experienced Demonstrators (**Figure S1E**; z=-0.329, $p = 0.996$; **Figure S1G**; z = 1.335, $p = 0.552$). There were also no differences in freezing or lever-pressing between Observers who witnessed either Demonstrator type in either shock pre-exposed (**Figure S1A vs. E**, light colors; z = 1.722, $p = 0.299$; **Figure S1C vs. G**, light colors; z=-2.094, $p = 0.137$) or shock-naïve Observers (**Figure S1A vs. E**, dark colors; z=-0.883, $p = 0.849$; **Figure S1C vs. G**, dark colors; z=-1.173, $p = 0.668$). Taken together, these results show that shock pre-exposure increased observational avoidance when Observers witness a Novice, but not an Experienced, Demonstrator.

To better understand the relationship between Demonstrator and Observer behaviors, we correlated the Observer's time on platform, freezing, and ITI lever-pressing at Test with their Demonstrator's behavior during the Observation Session. Correlating Observer and Demonstrator behaviors provides greater insight into how specific Demonstrator behaviors influence the Observer as opposed to general differences between Observer groups. Experienced Demonstrators exhibited increased avoidance compared to Novice Demonstrators (**Figure S2A**, time on platform, $p < 0.001$; **Figure S2B**, shocks avoided, $p < 0.001$) without showing differences in freezing (**Figure S2C**, $p = 0.289$). Time on platform was positively correlated between shock pre-exposed Observers and Novice Demonstrators (Fig. 2C, light purple triangles; $r = 0.484$, $p = 0.011$). Conversely, time on platform was negatively correlated between shock-naïve Observers and Novice Demonstrators (Fig. 2C, dark purple circles; r=-0.693, $p < 0.001$). Time on platform was not correlated between Observers and Experienced Demonstrators (Fig. 2F, light blue triangles; r=-0.040, $p = 0.865$; dark blue circles; $r = 0.272$, $p = 0.133$). There was a significant correlation in freezing between shock pre-exposed (**Figure S1B**, light purple triangles, $r = 0.588$, $p = 0.001$), but not shock-naïve, Observers and Novice Demonstrators (**Figure S1B**, dark purple circles, r=-0.048, $p = 0.823$). Conversely, shock-naïve Observers' freezing (**Figure S1F**, dark blue circles, $r = 0.482$, $p = 0.005$), but not shock pre-exposed Observers' freezing **(Figure S1F**, light blue triangles, $r = 0.265$, $p = 0.246$), was correlated with that of Experienced Demonstrators. ITI lever-pressing was not correlated between Observers and Novice (**Figure S1D**, light purple triangles, $r = 0.175$, $p = 0.384$; dark purple circles, r=-0.100, $p = 0.641$) or Experienced Demonstrators (**Figure S1H**, light blue triangles, $r = 0.175$, $p = 0.447$; dark blue circles, $r = 0.205$, $p = 0.261$). Taken together, these findings suggest that Observers' avoidance and freezing, but not lever-pressing, are influenced both by the Demonstrator's behavior and whether Observers have prior shock experience.

Shock pre-exposure enhances avoidance in male, but not female, Observers

Prior work has shown that females exhibit increased avoidance compared to males during direct learning of PMA[13]. We were therefore interested in whether there were any behavioral sex differences during observational PMA. Using the same data and models from Fig. 2, we conducted Sidak corrected planned contrasts to determine whether shock pre-exposure differentially impacts males and females. Shock pre-exposed males that witnessed a Novice Demonstrator spent significantly more time on the platform at Test compared to shock-naïve males (Fig. 3A, z=-2.953, $p = 0.013$); however, although it trended in the same direction, this was not the case for female Observers (Fig. 3C, z=-2.422, $p = 0.060$). Likewise, shock pre-exposure led to an increased number of shocks avoided in male (Fig. 3B, z=-3.345, $p = 0.003$), but not female Observers (Fig. 3D, z=-1.693, $p = 0.316$). After watching an Experienced Demonstrator, shock pre-exposure led to an increase in time on platform as well as the number of shocks avoided in male (Fig. 3G, z=-2.779, $p = 0.022$; Fig. 3H, z=-3.192, $p = 0.006$) but not female Observers (Fig. 3I, z = 0.532, $p = 0.973$; Fig. 3J z=-0.427, $p = 0.988$). Shock-naïve females also avoided more than shock naïve males after watching an Experienced Demonstrator (Fig. 3G and I, dark pink circles compared to dark green circles; z=-2.743, $p = 0.024$). Shock pre-exposure did not affect freezing in either sex when witnessing Novice (male, z=-2.428, $p = 0.072$; female, z=-0.914, $p = 0.797$) or Experienced Demonstrators (male, z = 0.315, $p = 0.989$; female, z = 0.162, $p = 0.999$).

Given that shock pre-exposure had a sex-dependent effect on avoidance, we were interested in how shock pre-exposure may have differentially affected correlations between Demonstrator and Observer avoidance in males and females. When witnessing Novice Demonstrators, time on platform was negatively correlated between shock-naïve males and the Demonstrator (Fig. 3E, dark green circles, r=-0.728, $p = 0.007$) and positively correlated between shock pre-exposed males and the Demonstrator (Fig. 3E, light green triangles, $r = 0.692$, $p = 0.003$). Whereas shock-naïve females exhibited the same behavioral correlation as the shock-naïve males (Fig. 3F, dark pink circles, r=-0.711, $p = 0.010$), the shock pre-exposed females showed no correlation with the Demonstrator's time on platform (Fig. 3F, light pink triangles, r=-0.030, $p = 0.930$). When witnessing an Experienced Demonstrator, time on platform was not correlated between the Observer and Demonstrator in either sex (Fig. 3K, dark green circles, $r = 0.016$, $p = 0.951$; light green triangles, r=-0.366, $p = 0.242$; Fig. 3L, dark pink circles, $r = 0.497$, $p = 0.070$; light pink triangles, $r = 0.558$, $p = 0.118$). Together with results from Fig. 2, these findings suggest that shock pre-exposure may be necessary for males, but not females, to effectively acquire Observational PMA.

Differences in Observational PMA acquisition cannot be explained by differences in visual attention

Prior work has shown that blocking visual information impairs observational learning[43–46], and we were thus interested in whether visual attention as measured by head orientation toward the barrier in front of the Demonstrator affected acquisition of Observational PMA (**Figure S3A**). There were no differences in orientation towards the Demonstrator between shock pre-exposed and shock-naïve Observers when witnessing a Novice (**Figure S3B**, z=-0.575, $p = 0.964$) or Experienced Demonstrator (**Figure S3H**, z=-1.487, $p = 0.445$). There were also no significant correlations between orientation and time on platform after witnessing a Novice Demonstrator (**Figure S3C**, light purple triangles, $r = 0.109$, $p = 0.596$; dark purple circles, $r = 0.157$, $p = 0.465$) or in shock-naïve Observers that witnessed an Experienced Demonstrator (**Figure S3I**, dark blue circles, r =

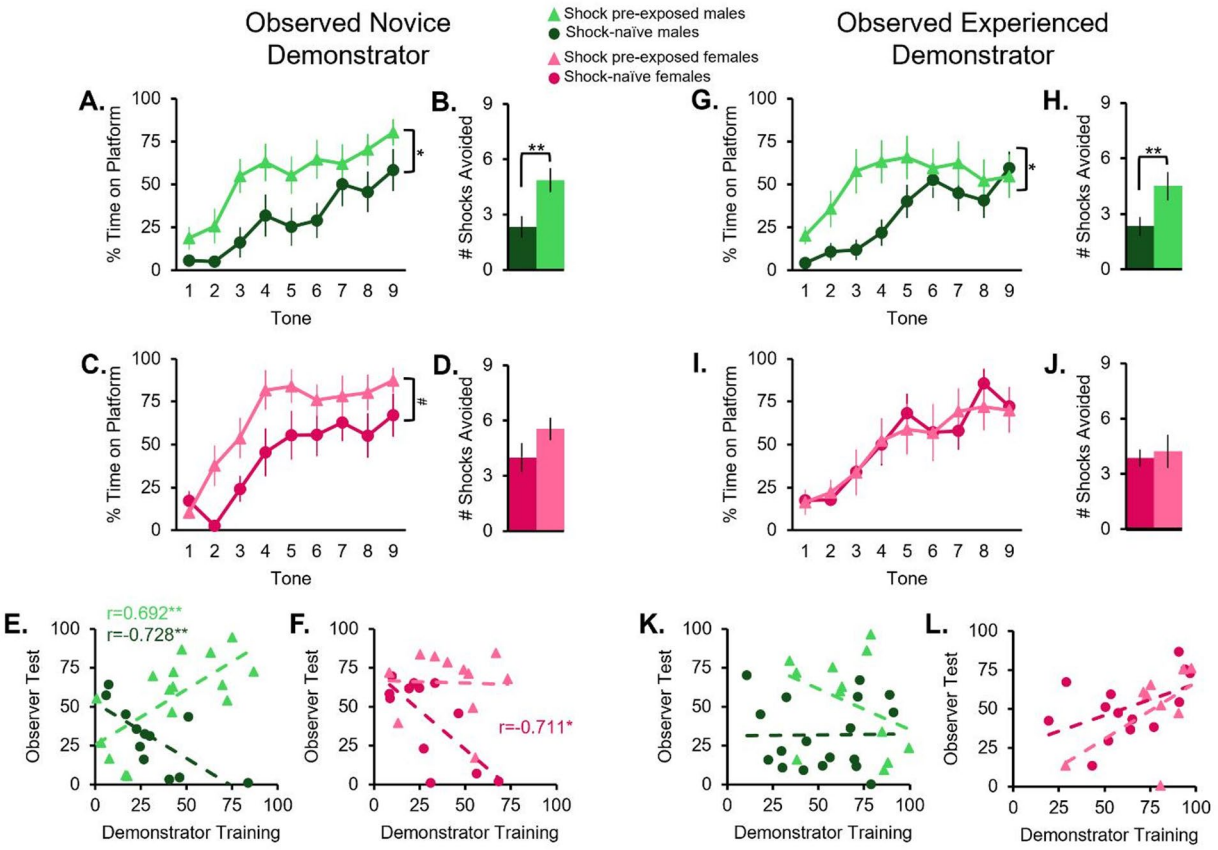

Fig. 3. Shock pre-exposure enhances avoidance in male, but not female, rats. (**A**) Shock pre-exposure increased male Observer time on platform ($z = -2.953$, $p = 0.013$) and (**B**) number of shocks avoided ($z = -3.345$, $p = 0.003$) after observing a Novice Demonstrator. (**C**) Shock pre-exposure did not alter female Observer time on platform ($z = -2.422$, $p = 0.060$) or (**D**) number of shocks avoided ($z = -1.693$, $p = 0.316$) after observing a Novice Demonstrator. (**E**) Male Observers' time on platform during the Test Session was positively correlated with their Demonstrator's time on platform during training for shock pre-exposed Observers (light green triangles, $r = 0.692$, $p = 0.003$) but negatively correlated for shock-naïve Observers (dark green circles, $r = -0.728$, $p = 0.007$). (**F**) Shock-naïve female Observers showed a negative correlation between their time on platform and that of their Demonstrator (dark pink circles, $r = -0.711$, $p = 0.010$), but there was no relationship for shock pre-exposed female Observers (light pink triangles, $r = -0.030$, $p = 0.930$). (**G**) Shock pre-exposure increased Male Observer time on platform ($z = -2.779$, $p = 0.022$) and (**H**) number of shocks avoided ($z = -3.192$, $p = 0.006$) after observing an Experienced Demonstrator. (**I**) Shock pre-exposure did not alter Female Observer time on platform ($z = 0.532$, $p = 0.973$) or (**J**) number of shocks avoided ($z = -0.427$, $p = 0.988$) after observing an Experienced Demonstrator. (**K**) There were no relationships between Observer and Demonstrator time on platform in either shock-naïve or shock pre-exposed male (dark green circles, $r = 0.016$, $p = 0.951$; light green triangles, $r = -0.366$, $p = 0.242$) or (**L**) female Observers (dark pink circles, $r = 0.497$, $p = 0.070$; light pink triangles, $r = 0.558$, $p = 0.118$). $n = 16$ shock pre-exposed males witnessing a Novice (light green), $n = 12$ shock-naïve males witnessing a Novice (dark green), $n = 11$ shock pre-exposed females witnessing a Novice (light pink), $n = 12$ shock-naïve females witnessing a Novice (dark pink), $n = 12$ shock pre-exposed males witnessing an Experienced Demonstrator (light green), $n = 18$ shock-naïve males witnessing an Experienced Demonstrator (dark green), $n = 9$ shock pre-exposed females witnessing an Experienced Demonstrator (light pink), $n = 14$ shock-naïve females witnessing an Experienced Demonstrator (dark pink). Data shown as mean and SEM. $\#p < 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$.

0.237, $p = 0.191$). However, orientation and time on platform were positively correlated in shock pre-exposed Observers that witnessed an Experienced Demonstrator (**Figure S3I**, light blue triangles, $r = 0.596$, $p = 0.004$). This positive correlation was driven by male Observers (**Figure S3K**, light green triangles, $r = 0.861$, $p < 0.001$). These results together with those from Fig. 3 suggest that male and female rats may utilize different information to acquire Observational PMA.

Observers exhibit three distinct behavioral phenotypes regardless of experimental condition

To better understand the different behavioral strategies that Observers may employ during Observational PMA, we conducted a hierarchical cluster analysis (see Methods). Previous work using a version of the PMA task has identified three behavioral phenotypes based on avoidance and lever-pressing[33]. We included avoidance (time

on platform and number of shocks avoided) and ITI lever-pressing in addition to freezing in our cluster analysis and identified three distinct behavioral phenotypes in the Observers regardless of their experimental condition (Fig. 4A).

Observers in Cluster 1 spent more time on the platform (Fig. 4B, Cluster 1, yellow, compared to 2, orange, $t = 13.37$, $p < 0.001$; Cluster 1, yellow, compared to 3, red, $t = 14.39$, $p < 0.001$) and avoided more shocks than the other clusters (Fig. 4C, Cluster 1, yellow, compared to 2, orange, $t = 15.37$, $p < 0.001$; Cluster 1, yellow, compared to 3, red, $t = 16.33$, $p < 0.001$) and were therefore labeled as Avoidance-Preferring Observers. Observers in Cluster 3 exhibited less freezing (Fig. 4D, Cluster 3, red, compared to 1, yellow, $t = 6.11$, $p < 0.001$; Cluster 3, red, compared to 2, orange, $t = 4.63$, $p < 0.001$) and performed a greater number of ITI lever-presses (Fig. 4E, Cluster 3, red, compared to 1, yellow, $t = 14.90$, $p < 0.001$; Cluster 3, red, compared to 2, orange, $t = 12.36$, $p < 0.001$) than the other clusters and were thus labeled as Reward-Preferring Observers. Observers in Cluster 2 were labeled as Balanced Behavior Observers because they showed levels of avoidance and lever-pressing between those of the Avoidance-Preferring and Reward-Preferring Observers.

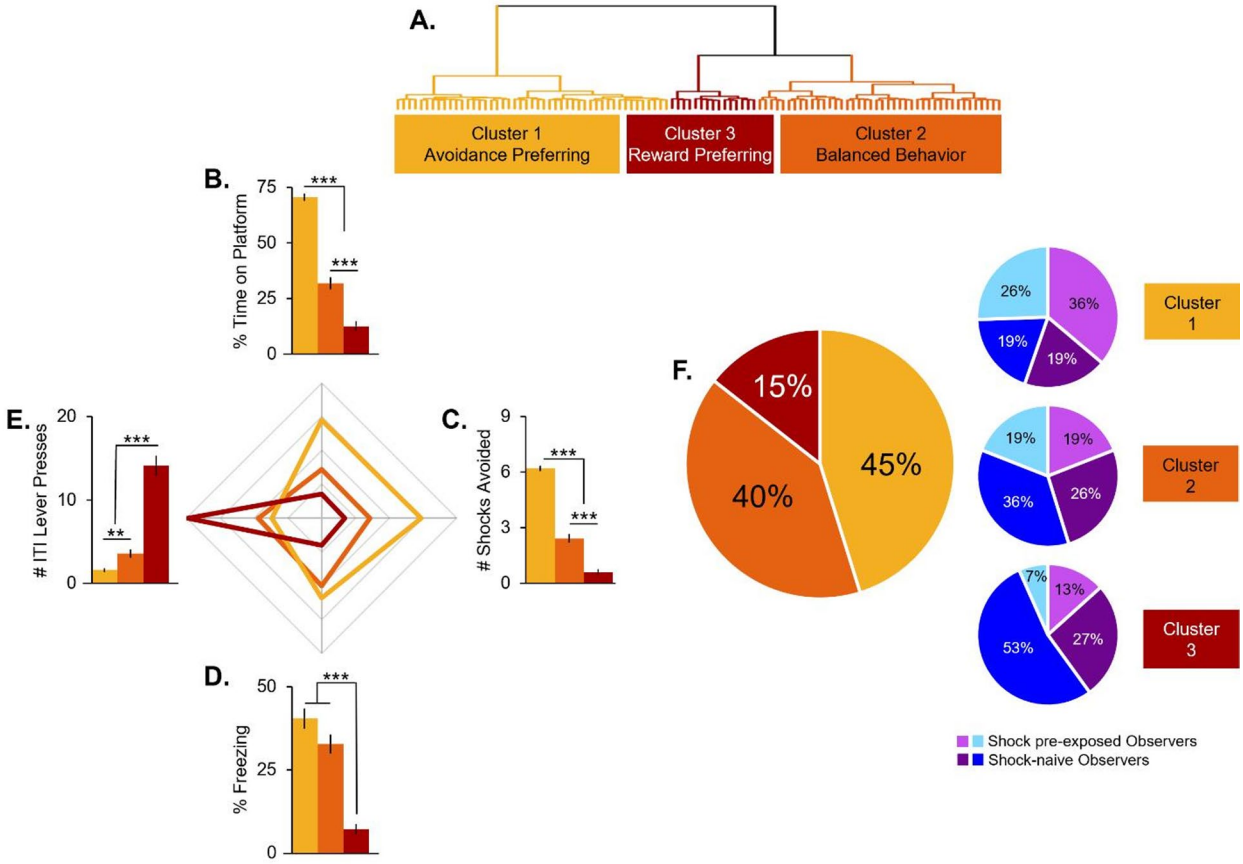

Fig. 4. Shock pre-exposed Observers adopt an avoidance-preferring strategy whereas shock-naïve Observers adopt a reward-preferring strategy. (**A**) Dendrogram of behavioral clusters. (**B**) Avoidance-Preferring Observers ($n = 40$) spent more time on the platform than the other clusters (Balanced Behavior, $t = 13.37$, $p < 0.001$; Reward-Preferring, $t = 14.39$, $p < 0.001$), and Balanced Behavior Observers ($n = 39$) spent more time on the platform than Reward-Preferring Observers ($n = 14$) ($t = 4.74$, $p < 0.001$). (**C**) Avoidance-Preferring Observers avoided more shocks than the other clusters (Balanced Behavior, $t = 15.37$, $p < 0.001$; Reward-Preferring, $t = 16.33$, $p < 0.001$), and Balanced Behavior Observers avoided more shocks than Reward-Preferring Observers ($t = 5.24$, $p < 0.001$). (**D**) Reward-Preferring Observers froze significantly less than the other clusters (Avoidance-Preferring, $t = 6.11$, $p < 0.001$; Balanced Behavior, $t = 4.63$, $p < 0.001$). (**E**) Reward-Preferring Observers made significantly more lever presses than the other clusters (Avoidance-Preferring, $t = 14.90$, $p < 0.001$; Balanced Behavior, $t = 12.36$, $p < 0.001$). Balanced Behavior Observers lever-pressed more than Avoidance-Preferring Observers ($t = 3.30$, $p = 0.004$). Center radar plot depicts the behavioral profile of each cluster across the four measures. Variables were scaled to be comparable within a range from −2 to 2. (**F**) Shock pre-exposure and behavioral cluster were not independent (Fisher's Exact Test, $p = 0.006$). There were more shock pre-exposed Observers in the Avoidance-preferring cluster ($n = 47$, yellow, *top*) and fewer shock pre-exposed Observers in the Reward-preferring cluster ($n = 15$, red, *bottom*) than expected while the number of Balanced Behavior Observers did not deviate from expectation ($n = 42$, orange, *middle*). Data shown as mean and SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.

We were next interested in exploring the composition of the three behavioral clusters in terms of experimental groups. We used a Fisher's Exact Test and determined that an Observer's behavioral cluster was dependent on their experience with footshock prior to the Test Session ($p = 0.006$). The Avoidance-Preferring cluster was comprised of moderately more shock pre-exposed Observers (standardized residual = 1.57) and fewer shock-naïve Observers (standardized residual=-1.45) than expected (Fig. 4F, top right). Conversely, the Reward-Preferring cluster was comprised of moderately fewer shock pre-exposed (standardized residual=-1.49) and more shock-naïve Observers (standardized residual = 1.38) than expected (Fig. 4F, bottom right). These results suggest that shock pre-exposure led Observers to adopt an avoidance-focused strategy during Observational PMA as opposed to a reward-focused strategy or a balance between the two.

Because we found both sex differences (Fig. 3) and differences in behavioral clusters following shock pre-exposure (Fig. 4), we further divided the clusters by sex (Fig. 5). A Fisher's Exact Test revealed that sex and behavioral cluster were dependent upon each other ($p = 0.021$). This effect was moderately driven by Cluster 3 in which there were fewer females (standardized residual=-1.80) and more males (standardized residual = 1.60) than expected (Fig. 5A left compared to **B** left). We next ran Fisher's Exact Tests within each sex and found that experience with footshock and behavioral cluster were dependent in male Observers (Fig. 5A, $p = 0.010$). This effect was moderately driven by an increased number of shock pre-exposed (standardized residual = 1.65) and a decreased number of shock-naïve Observers (standardized residual=-1.59) in the Avoidance-Preferring cluster (Fig. 5A, top right, light blue and purple (27 + 41 = 68%) compared to dark blue and purple (23 + 9 = 32%)). There were also fewer shock pre-exposed (standardized residual=-1.31) and more shock-naïve Observers (standardized residual = 1.26) than expected in Cluster 1 (Fig. 5A, bottom right, light blue and purple (8 + 15 = 23%) compared to dark blue and purple (54 + 23 = 77%)).Conversely, experience with footshock and behavioral cluster were independent in female Observers (Fig. 5B, $p = 0.364$). These results show that the behavioral phenotype of male, but not female, Observers was dependent upon their experience with footshock. Shock pre-exposure led males to adopt the Avoidance-Preferring strategy when they may otherwise have adopted the Reward-Preferring strategy, but females had a high percentage of Avoidance-Preferring individuals, regardless of prior shock experience.

mPFC connectivity extends to subcortical circuits when Observers witnessed an Experienced Demonstrator

We next wanted to gain insight into the neural substrates guiding Observational PMA using c-Fos immunohistochemistry (Fig. 6A). The ACC is a central hub for observational learning[14,24,46–49] and shares

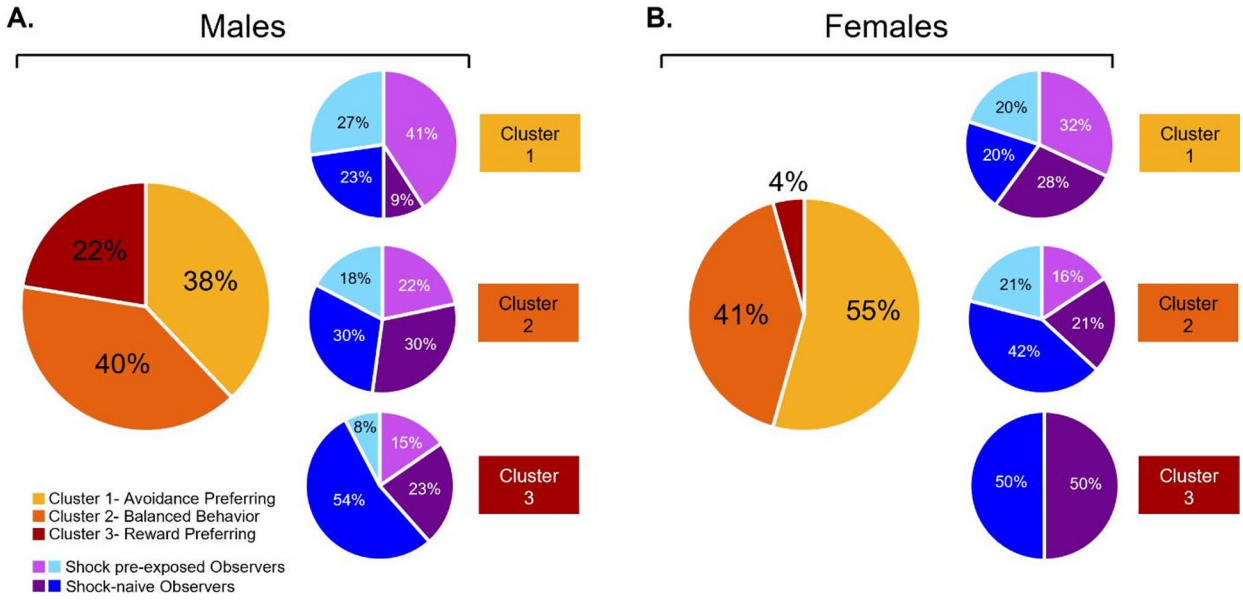

Fig. 5. Shock pre-exposure shifts male, but not female, behavioral strategies toward the avoidance-preferring strategy. (**A**) *Left*: Proportion of male Observers in each behavioral cluster. Sex and behavioral cluster were dependent upon each other (Fisher's Exact Test, $p = 0.021$), and this was driven by more males and fewer females than expected falling into Cluster 3. *Right*: Shock pre-exposure and behavioral cluster were dependent upon each other in male Observers (Fisher's Exact Test, $p = 0.010$). There were more shock pre-exposed male Observers in the Avoidance-preferring cluster and fewer shock pre-exposed male Observers in the Reward-preferring cluster than expected. Avoidance-Preferring males ($n = 22$, yellow, *top*) were mostly shock pre-exposed while Balanced Behavior ($n = 23$, orange, *middle*) and Reward-Preferring ($n = 13$, red, *bottom*) males were mostly shock-naïve. (**B**) *Left*: Proportion of female Observers in each behavioral cluster. *Right*: Shock pre-exposure and behavioral cluster were independent in female Observers (Fisher's Exact Test, $p = 0.364$). Avoidance-Preferring females ($n = 25$, yellow, *top*) were evenly distributed between shock-naïve and shock pre-exposed. Balanced behavior ($n = 19$, orange, *middle*) females were mostly shock-naïve. There were no shock pre-exposed females that were Reward-Preferring ($n = 2$, red, *bottom*).

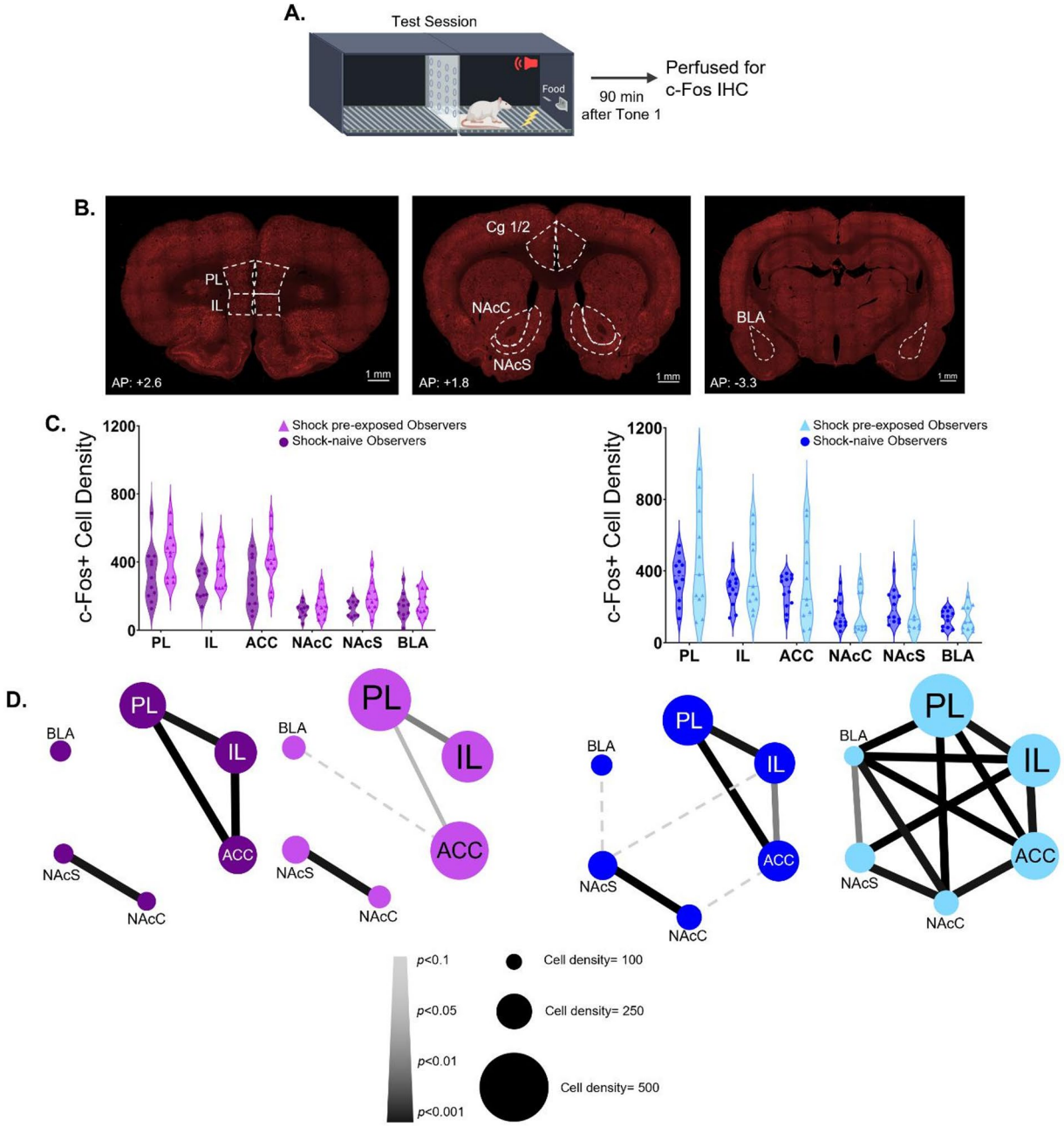

Fig. 6. Observers exhibit distinct neural activation patterns that depend on Demonstrator experience and Observer's shock experience. (**A**) Schematic showing Test Session and perfusion timeline. (**B**) Example images of cFos quantification in PL and IL (*left*), Cg 1/2 (part of ACC), NAcC, and NAcS (*middle*), and the BLA (*right*). (**C**) There were no differences in c-Fos Density between shock-naïve and shock pre-exposed Observers who watched a Novice Demonstrator (*left*, PL, $t = 1.440$, $p = 0.496$; IL, $t = 1.183$, $p = 0.673$; ACC, $t = 2.128$, $p = 0.149$; NAcC, $t = 0.871$, $p = 0.861$; NAcS, $t = 1.243$, $p = 0.632$; BLA, $t = 0.837$, $p = 0.877$) or an Experienced Demonstrator (*right*, PL, $t = -1.027$, $p = 0.774$; IL, $t = -1.345$, $p = 0.555$; ACC, $t = -0.653$, $p = 0.946$; NAcC, $t = 0.176$, $p = 0.999$; NAcS, $t = -0.054$, $p = 0.999$; BLA, $t = 0.405$, $p = 0.991$). (**D**) Shock-naïve Observers that watched a Novice Demonstrator showed stronger correlations in activation between medial prefrontal subregions (*left*) than shock pre-exposed Observers (*left middle*). After watching an Experienced Demonstrator, shock pre-exposed Observers (*right*) showed more correlations in activation between brain areas than shock-naïve (*right middle*) Observers. $n = 11$ shock pre-exposed Observers witnessing a Novice (light purple), $n = 11$ shock-naïve Observers witnessing a Novice (dark purple),, $n = 11$ shock pre-exposed Observers witnessing an Experienced Demonstrator (light blue), $n = 12$ shock-naïve Observers witnessing an Experienced Demonstrator (dark blue). Width and color of lines indicate significance level of correlation. Dotted lines indicate a trending correlation ($p < 0.100$).

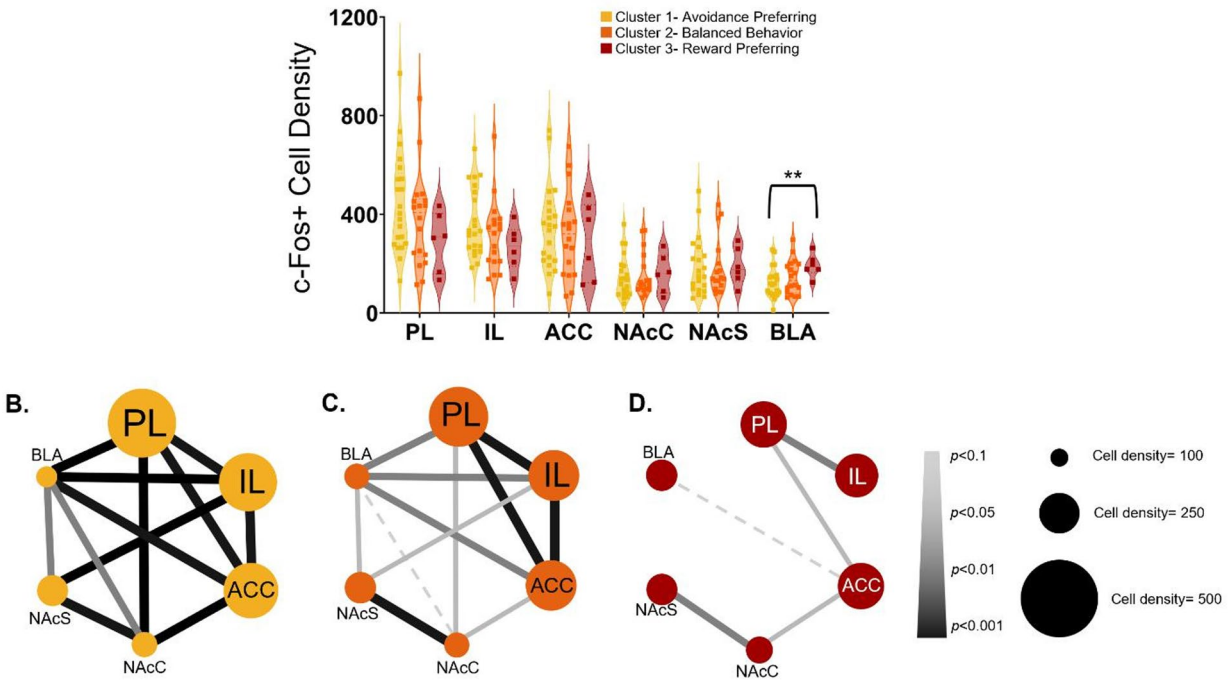

Fig. 7. Avoidance-Preferring and Balanced Behavior Observers show similar neural activation profiles that are more correlated compared to Reward-Preferring Observers. (**A**) There were no differences in neural activation between behavioral clusters (PL: 1 vs. 2 t = 0.629, $p = 0.898$, 2 vs. 3 t = − 0.251, $p = 0.992$; 1 vs. 3 t = − 0.694, $p = 0.869$; IL: 1 vs. 2, t = 0.332, $p = 0.983$, 2 vs. 3, t = − 0.483, $p = 0.950$; 1 vs. 3, t = − 0.721, $p = 0.855$; ACC: 1 vs. 2, t = 1.359, $p = 0.452$, 2 vs. 3, t = − 0.739, $p = 0.846$, 1 vs. 3, t = − 1.697, $p = 0.264$; NAcC: 1 vs. 2, t = 0.470, $p = 0.954$, 2 vs. 3, t = − 0.682, $p = 0.874$; 1 vs. 3, t = − 1.029, $p = 0.671$; NAcS: 1 vs. 2, t = 0.691, $p = 0.870$, 2 vs. 3, t = − 0.244, $p = 0.993$; 1 vs. 3, t = − 0.739, $p = 0.846$; BLA: 1 vs. 2, t = 1.962, $p = 0.160$, 2 vs. 3, t = − 1.769, $p = 0.232$; 1 vs. 3, t = − 3.161, $p = 0.009$). Correlations in neural activation for (**B**) Avoidance-Preferring, (**C**) Balanced Behavior, and (**D**) Reward-Preferring Observers. $n = 21$ Avoidance-Preferring (yellow), $n = 18$ Balanced Behavior (orange), $n = 6$ Reward-Preferring (red). Width and color of lines indicate significance level of correlation. Dotted lines indicate a trending correlation ($p < 0.100$).

connections with the direct PMA circuit through its projections to the nucleus accumbens core (NAcC)[21] and BLA[17,18]. The IL is necessary for avoidance responses during direct learning of both shuttle[7–9] and lever-press avoidance[10,11] and projects to the BLA[19] and the shell of the nucleus accumbens (NAcS)[20]. Furthermore, the BLA projects to both the NAcC and NAcS[50], creating a map of areas that are potentially activated during Observational PMA.

There were no differences in c-Fos density between shock-naïve and shock pre-exposed Observers who witnessed either a Novice or an Experienced Demonstrator for any of the brain regions (PL, IL, ACC, NAcC, NAcS, BLA; Fig. 6C). To visualize neural activation profiles during Observational PMA, we correlated c-Fos densities of the brain regions for each experimental group. After witnessing a Novice Demonstrator, shock-naïve Observers showed stronger correlations in activation between medial prefrontal subregions (PL, IL, ACC; Fig. 6D, left; **Figure S4A**) compared to shock pre-exposed Observers (Fig. 6D left middle, **Figure S4B)**. After witnessing an Experienced Demonstrator, shock-naïve Observers exhibited significant correlations between medial prefrontal subregions (Fig. 6D, right middle; **Figure S4C**) similar to those observed in shock-naïve Observers that witnessed a Novice Demonstrator. Shock pre-exposed Observers, however, exhibited positive correlations between all anatomically connected areas (Fig. 6D, right; **Figure S4D**).

We then examined differences in neural activation between behavioral clusters in a similar manner. BLA activation was significantly increased in Reward-Preferring compared to Avoidance-Preferring Observers (Fig. 7A, t=-3.161, $p = 0.009$), but no other differences in neural activation were observed. However, neural activation profiles varied greatly between clusters. The Avoidance-Preferring Observers showed positive correlations between all anatomically connected brain regions (Fig. 7B; **Figure S4E**). Likewise, the Balanced Behavior Observers were only lacking one positive correlation between the NAcC and BLA (Fig. 7C; **Figure S4F**). The Reward-Preferring Observers showed fewer correlations than either the Avoidance-Preferring or Balanced Behavior Observers. Notably, the Reward-Preferring Observers showed no significant correlations in activation involving the BLA despite showing increased activation in the BLA compared to Avoidance-Preferring Observers (Fig. 7D; **Figure S4G**).

Discussion

We developed a novel task for the study of observational active avoidance. Overall, we found that Observers' avoidance was affected by several factors including sex, exposure to footshock, and Demonstrator experience. Additionally, we examined neural activation in several brain areas previously implicated in avoidance. Although there were minimal differences in activation across Observer groups, each Observer group displayed neural activation profiles. We discuss below how Observers witnessing Demonstrators with different experience levels may utilize different social information and may recruit distinct neural circuits to acquire active avoidance as well as provide future directions for directly testing these hypotheses.

We exposed half of the Observers to footshock prior to the Observation Session, as previous studies on observational avoidance had not used both shock pre-exposed and shock-naïve Observers in the same study[2–4]. In addition, previous observational fear learning (OFL) studies are inconsistent in the use of shock pre-exposure. Some studies find evidence that experience with footshock is necessary for OFL[22–24,51], while others only include shock-naïve[46,48,52–55] or shock pre-exposed Observers[56]. These discrepancies in methodology leave much about the effects of shock pre-exposure on learning unclear. In previous OFL studies that included both groups, shock-naïve Observers did not increase their freezing from baseline during a recall session 24 h later, indicating that they did not acquire OFL to the same extent as shock pre-exposed Observers[22,24]. A similar effect was noted in the present study when Observers witnessed a Novice Demonstrator: shock-naïve Observers did not increase their avoidance to the same extent as shock pre-exposed Observers during their Test Session (Fig. 2A). Shock pre-exposure improving both OFL and Observational PMA when witnessing a Novice Demonstrator may be explained by behavioral similarities between classical fear conditioning and early stages of PMA training. Prior work using the PMA task shows that early stages of training resemble classical fear conditioning as rats have not yet learned to actively avoid a tone-signaled footshock and instead freeze[12,13,41,57,58].

Unlike OFL studies, however, we found that shock pre-exposure increased avoidance in male Observers (Fig. 3A-D). Previous OFL studies have found that both male[23,24,51] and female[22,23] shock pre-exposed Observers exhibit increased freezing compared to shock-naïve Observers. Our findings are consistent with the idea that females avoid more than males when PMA is learned through direct experience[13]. Because females are predisposed to increased avoidance, observation alone may be sufficient for them to acquire PMA whereas males require shock pre-exposure to acquire avoidance through observation. This explanation is further supported by the findings that shock pre-exposure increased avoidance in males that observed an Experienced Demonstrator (Fig. 3G-J) and caused males to adopt an Avoidance-Preferring over a Reward-Preferring behavioral strategy during the Test Session (Fig. 5A).

In addition to influencing Observers' behavioral strategies at Test (Figs. 4F and 5A), shock pre-exposure altered relationships between Demonstrator and Observer behaviors. Shock pre-exposed Observers spent more time on the platform when their Novice Demonstrator did as well. This relationship was reversed in shock-naïve Observers (Fig. 2C). Novice Demonstrators were not yet familiar with the task and received more footshocks compared to Experienced Demonstrators (**Figure S2B**). When a shock pre-exposed Observer witnessed a Novice Demonstrator receive shock, the aversive reactions may have been familiar to them due to auto-conditioning[51,59,60]. Auto-conditioning occurs when rodents associate their aversive reactions (often vocalizations) with distress and these associations later enhance OFL[51,59,60]. The lack of aversive reactions when a Demonstrator avoided shock can therefore be informative to shock pre-exposed Observers, leading the Observer to avoid more during their Test Session. Shock-naïve Observers had no context for the Demonstrator's aversive reactions to shock and likely needed to observe more shock trials (less avoidance) to understand the motivation of the avoidance response, leading to a negative correlation between Demonstrator and Observer avoidance. These effects were also largely driven by male Observers who required shock pre-exposure to acquire PMA. Future work could include ultrasonic vocalization monitoring to further investigate the auto-conditioning hypothesis as well as further investigate sex differences in Observational PMA.

In contrast to observing a Novice Demonstrator, Observers that witnessed an Experienced Demonstrator showed no correlation between their avoidance and that of their Demonstrator (Fig. 2F). This suggests that other social information may be used to acquire Observational PMA. Experienced Demonstrators avoided more shocks compared to Novice Demonstrators (**Figure S2B**), so effects from auto-conditioning during shock pre-exposure likely did not aid in Observational PMA acquisition. Instead, we found that shock-naïve Observers showed more freezing as Experienced Demonstrators showed more freezing (**Figure S1F**). Prior work has shown that silence and lack of movement caused by freezing are informative danger signals within a social context[61]. Given that Experienced Demonstrators show high avoidance and low freezing, freezing may be more salient and thus convey more information than the avoidance response. Shock pre-exposed Observers that visually attended more to an Experienced Demonstrator also avoided more during their Test Session (**Figure S3I**), further suggesting that social information other than Demonstrator avoidance promotes Observational PMA acquisition. Future work could determine which Demonstrator behaviors trigger Observer visual attention as well as which auditory signals influence Observational PMA acquisition.

We did not find differences in neural activation across Observer groups (Fig. 6), suggesting that neither shock pre-exposure nor observing an Experienced Demonstrator is associated with increases in neural activation in areas necessary for active avoidance. We did, however, find a significant increase in BLA activation in the Reward-Preferring cluster compared to the Avoidance-Preferring cluster (Fig. 7). The BLA is well-known for its role in aversive learning[58,62,63], but the BLA is also important for learning the value of a reward[64]. Increased BLA activation may therefore indicate an increased value assigned to sucrose by Reward-Preferring Observers. Importantly, this finding cannot be attributed to differences in hunger levels because all Observers were food-restricted according to weight during experiments.

The lack of differences in neural activation may have resulted from a variety of factors including task complexity and limitations of c-Fos IHC. Observers performed a variety of behaviors throughout the Test Session, and c-Fos

IHC lacks the temporal precision[31,65] needed to determine which specific behaviors may be tied to activation. There is also a range of time in which protein expression peaks (60–120 minutes[31], so it is possible that we captured fluctuations in activation that represent multiple behaviors that occurred during the Test. Future work could employ techniques such as single-unit electrophysiology or fiber photometry to determine which Observer behaviors are tied to changes in activation in these areas. Finally, c-Fos is also not cell-type specific[65]. Future work could utilize c-Fos co-labeling to determine which cell types are activated during Observational PMA or combine anatomical tract tracing with c-Fos IHC to determine which projections are activated during Observational PMA. Overall, the use of this method was intended to provide an initial framework for future investigations into the necessary circuitry for observational avoidance. The information gained from this study can help narrow the areas of interest for future studies using neural recordings or manipulations to determine the circuits necessary for observational avoidance.

While we did not investigate specific projections in this study, we correlated neural activation between areas and found that Observers exhibited unique activation profiles when classified by experimental group or behavioral cluster (Figs. 6 and 7). Observers of Novice Demonstrators exhibited similar activation profiles regardless of shock pre-exposure. Activation profiles differed greatly, however, between shock-naïve and shock pre-exposed Observers of Experienced Demonstrators. This effect can potentially be explained by a combination of the complexity of the task that Observers witnessed at this timepoint and that the shock pre-exposure from the prior day may have acted as an acute stressor that increased brain-wide plasticity and led to changes in the activation profile[66,67]. Future work should examine neural activation during both the Observation and Test Session to determine whether the same or separate neural populations are responsible for observing and executing avoidance.

The Avoidance-Preferring Observers exhibited more correlations in activation between brain areas known to constitute the direct PMA circuit (PL, NAcC, BLA)[5]. The activation profile of the Balanced Behavior Observers notably lacked a correlation between BLA and NAcC, a projection known to promote avoidance expression in PMA[5]. Furthermore, the activation profile of the Reward-Preferring Observers did not exhibit correlations between any of the areas of the direct PMA circuit. Given that two of the three clusters showed a positive correlation between PL and NAcC, a projection known to inhibit avoidance expression in PMA[5], future work could investigate whether the density of this projection or the level of activation of this projection differs between behavioral clusters. Alternatively, future work could manipulate PL-NAcC projection neurons using optogenetics or chemogenetics to determine whether it has the same role in Observational PMA as in direct learning of PMA[5] and whether the role of this projection differs between clusters.

We were also intrigued by the correlations involving IL observed in multiple groups given that this area is not necessary for avoidance expression in PMA[12]. IL is, however, implicated in shuttle[6–9] and lever-press avoidance[10,11,58,68]. In the shock pre-exposed Observers, IL may be suppressing freezing responses learned on the previous day to perform avoidance due to its role in inhibiting previously learned information to allow the formation of new stimulus-outcome associations[11,69–72]. Likewise, IL may also be suppressing freezing or other behaviors that could interfere with avoidance in the Avoidance-Preferring and Balanced Behavior clusters. Future work should investigate whether IL activation is necessary for avoidance expression in Observational PMA to determine how the neural mechanisms of observational learning differ from those of direct PMA acquisition.

Taken together, this work furthers our understanding of observational avoidance learning by comparing the effects of Demonstrator experience, exposure to shock, and sex within a single study. Interestingly, we found that Observers rely on different sources of information depending on their sex and Demonstrator type. Findings from this study also add to the growing body of literature investigating sex differences in avoidance learning. Future work using the Observational PMA task should investigate additional aspects such as darting and USV emission to further understand why males and females acquire avoidance differently. This work also provides insight into neural activation during Observational PMA and how activation profiles differ depending on shock pre-exposure and Demonstrator experience. Future studies will investigate the circuitry necessary for Observational PMA in order to understand how individuals may learn complex and adaptive tasks, like active avoidance, by observing others.

Data availability

Data will be made available upon request.

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References

1. Thorndike, E. L. Animal intelligence: An experimental study of the associative processes in animals. *Psychol. Rev. Monogr. Suppl.* **2**, i–109 (1898).
2. Del Russo, J. E. Observational learning of discriminative avoidance in hooded rats. *Anim. Learn. Behav.* **3**, 76–80 (1975).
3. Presley, W. J. & Riopelle, A. J. Observational Learning of an Avoidance Response. *J. Genet. Psychol.* **95**, 251–254 (1959).
4. John, E. R., Chesler, P., Bartlett, F. & Victor, I. Observation Learning in Cats. *Science* **159**, 1489–1491 (1968).
5. Diehl, M. M. et al. Divergent projections of the prelimbic cortex bidirectionally regulate active avoidance. *eLife* **9**, e59281 (2020).
6. Ramirez, F., Moscarello, J. M., LeDoux, J. E. & Sears, R. M. Active Avoidance Requires a Serial Basal Amygdala to Nucleus Accumbens Shell Circuit. *J. Neurosci.* **35**, 3470–3477 (2015).
7. Martinez, R. C. R. et al. Active vs. reactive threat responding is associated with differential c-Fos expression in specific regions of amygdala and prefrontal cortex. *Learn. Mem.* **20**, 446–452 (2013).
8. Moscarello, J. M. & LeDoux, J. E. Active Avoidance Learning Requires Prefrontal Suppression of Amygdala-Mediated Defensive Reactions. *J. Neurosci.* **33**, 3815–3823 (2013).
9. Cain, C. K. Avoidance problems reconsidered. *Curr. Opin. Behav. Sci.* **26**, 9–17 (2019).

10. Jiao, X., Beck, K. D., Myers, C. E., Servatius, R. J. & Pang, K. C. H. Altered activity of the medial prefrontal cortex and amygdala during acquisition and extinction of an active avoidance task. *Front Behav. Neurosci* **9**, (2015).
11. Capuzzo, G. & Floresco, S. B. Prelimbic and Infralimbic Prefrontal Regulation of Active and Inhibitory Avoidance and Reward-Seeking. *J. Neurosci.* **40**, 4773–4787 (2020).
12. Bravo-Rivera, C., Roman-Ortiz, C., Brignoni-Perez, E., Sotres-Bayon, F. & Quirk, G. J. Neural Structures Mediating Expression and Extinction of Platform-Mediated Avoidance. *J. Neurosci.* **34**, 9736–9742 (2014).
13. Ruble, S. *et al. Social Context Modulates Active Avoidance: Contributions of the Anterior Cingulate Cortex in Male and Female Rats.* (2025). https://doi.org/10.1016/j.ynstr.2024.100702
14. Burgos-Robles, A., Gothard, K. M., Monfils, M. H., Morozov, A. & Vicentic, A. Conserved features of anterior cingulate networks support observational learning across species. *Neurosci. Biobehav Rev.* **107**, 215–228 (2019).
15. Keum, S. & Shin, H. S. Neural Basis of Observational Fear Learning: A Potential Model of Affective Empathy. *Neuron* **104**, 78–86 (2019).
16. Lindström, B., Haaker, J. & Olsson, A. A common neural network differentially mediates direct and social fear learning. *NeuroImage* **167**, 121–129 (2018).
17. Gabbott, P. L. A., Warner, T. A., Jays, P. R. L., Salway, P. & Busby, S. J. Prefrontal cortex in the rat: Projections to subcortical autonomic, motor, and limbic centers. *J. Comp. Neurol.* **492**, 145–177 (2005).
18. Bissière, S. et al. The Rostral Anterior Cingulate Cortex Modulates the Efficiency of Amygdala-Dependent Fear Learning. *Biol. Psychiatry*. **63**, 821–831 (2008).
19. Li, Z. et al. Input–output organization of the mouse BLA-projecting IL neurons. *Front. Neurosci.* **19**, 1532078 (2025).
20. Brog, J. S., Salyapongse, A., Deutch, A. Y. & Zahm, D. S. The patterns of afferent innervation of the core and shell in the Accumbens part of the rat ventral striatum: Immunohistochemical detection of retrogradely transported fluoro-gold. *J. Comp. Neurol.* **338**, 255–278 (1993).
21. Smith, M. L., Asada, N. & Malenka, R. C. Anterior cingulate inputs to nucleus accumbens control the social transfer of pain and analgesia. *Science* **371**, 153–159 (2021).
22. Atsak, P. et al. Experience Modulates Vicarious Freezing in Rats: A Model for Empathy. *PLoS ONE*. **6**, e21855 (2011).
23. Sanders, J., Mayford, M. & Jeste, D. Empathic Fear Responses in Mice Are Triggered by Recognition of a Shared Experience. *PLoS ONE*. **8**, e74609 (2013).
24. Allsop, S. A. et al. Corticoamygdala Transfer of Socially Derived Information Gates Observational Learning. *Cell* **173**, 1329–1342e18 (2018).
25. Kim, A., Keum, S. & Shin, H. Observational fear behavior in rodents as a model for empathy. *Genes Brain Behav.* **18**, e12521 (2019).
26. Kramer, C., Ruble, S., Fort, T. D., West, L. & Diehl, M. M. Modifying the platform-mediated avoidance task: A new protocol to study active avoidance within a social context in rats. *PLOS One*. **20**, e0321776 (2025).
27. Nikbakht, N. & Diamond, M. E. Conserved visual capacity of rats under red light. *eLife* **10**, e66429 (2021).
28. Wallace, D. J. et al. Rats maintain an overhead binocular field at the expense of constant fusion. *Nature* **498**, 65–69 (2013).
29. Bullitt, E. Expression of *C - fos* -like protein as a marker for neuronal activity following noxious stimulation in the rat. *J. Comp. Neurol.* **296**, 517–530 (1990).
30. Chung, L. A Brief Introduction to the Transduction of Neural Activity into Fos Signal. *Dev. Reprod.* **19**, 61–67 (2015).
31. Kovács, K. J. Measurement of Immediate-Early Gene Activation- *c-fos* and Beyond. *J. Neuroendocrinol.* **20**, 665–672 (2008).
32. Silva, B. A., Burns, A. M. & Gräff, J. A cFos activation map of remote fear memory attenuation. *Psychopharmacol. (Berl).* **236**, 369–381 (2019).
33. Bravo-Rivera, H. et al. Characterizing Different Strategies for Resolving Approach-Avoidance Conflict. *Front. Neurosci.* **15**, 608922 (2021).
34. De Paula Cunha Almeida, C. et al. Prefrontal and Subcortical c-Fos Mapping of Reward Responses across Competitive and Social Contexts. *eneuro* **12**, ENEURO0158–252025 (2025).
35. Felix-Ortiz, A. C., Burgos-Robles, A., Bhagat, N. D., Leppla, C. A. & Tye, K. M. Bidirectional modulation of anxiety-related and social behaviors by amygdala projections to the medial prefrontal cortex. *Neuroscience* **321**, 197–209 (2016).
36. Bankhead, P. et al. QuPath: Open source software for digital pathology image analysis. *Sci. Rep.* **7**, 16878 (2017).
37. Brooks, M. glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. *R J.* **9**, 378 (2017).
38. McGillycuddy, M., Warton, D. I., Popovic, G. & Bolker, B. M. Parsimoniously Fitting Large Multivariate Random Effects in glmmTMB. *J Stat. Softw* **112**, (2025).
39. Kuhn, M., Jackson, S. & Cimentada J. corrr: Correlations in R. (2022).
40. Lenth, R. & Piaskowski, J. *Estimated Marginal Means, aka Least-Squares Means* (R package, 2025).
41. Diehl, M. M., Bravo-Rivera, C. & Quirk, G. J. The study of active avoidance: A platform for discussion. *Neurosci. Biobehav Rev.* **107**, 229–237 (2019).
42. Martínez-Rivera, F. J., Bravo-Rivera, C., Velázquez-Díaz, C. D., Montesinos-Cartagena, M. & Quirk, G. J. Prefrontal circuits signaling active avoidance retrieval and extinction. *Psychopharmacol. (Berl).* **236**, 399–406 (2019).
43. Groesbeck, R. W. & Duerfeldt, P. H. Some relevant variables in observational learning of the rat. *Psychon Sci.* **22**, 41–43 (1971).
44. Hong, E. H. & Choi, J. S. Observational threat conditioning is induced by circa-strike activity burst but not freezing and requires visual attention. *Behav. Brain Res.* **353**, 161–167 (2018).
45. Kavaliers, M., Choleris, E. & Colwell, D. D. Learning from others to cope with biting flies: Social learning of fear-induced conditioned analgesia and active avoidance. *Behav. Neurosci.* **115**, 661–674 (2001).
46. Jeon, D. et al. Observational fear learning involves affective pain system and Cav1.2 Ca2 + channels in ACC. *Nat. Neurosci.* **13**, 482–488 (2010).
47. Kim, S., Mátyás, F., Lee, S., Acsády, L. & Shin, H. S. Lateralization of observational fear learning at the cortical but not thalamic level in mice. *Proc. Natl. Acad. Sci.* 109, 15497–15501 (2012).
48. Keum, S. et al. A Missense Variant at the Nrxn3 Locus Enhances Empathy Fear in the Mouse. *Neuron* **98**, 588–601e5 (2018).
49. Carrillo, M. et al. Emotional Mirror Neurons in the Rat's Anterior Cingulate Cortex. *Curr. Biol.* **29**, 1301–1312e6 (2019).
50. Shiflett, M. W. & Balleine, B. W. At the limbic–motor interface: disconnection of basolateral amygdala from nucleus accumbens core and shell reveals dissociable components of incentive motivation. *Eur. J. Neurosci.* **32**, 1735–1743 (2010).
51. Kim, E. J., Kim, E. S., Covey, E. & Kim, J. J. Social Transmission of Fear in Rats: The Role of 22-kHz Ultrasonic Distress Vocalization. *PLoS ONE*. **5**, e15077 (2010).
52. Twining, R. C., Vantrease, J. E., Love, S., Padival, M. & Rosenkranz, J. A. An intra-amygdala circuit specifically regulates social fear learning. *Nat. Neurosci.* **20**, 459–469 (2017).
53. Yusufishaq, S. & Rosenkranz, J. A. Post-weaning social isolation impairs observational fear conditioning. *Behav. Brain Res.* **242**, 142–149 (2013).
54. Chen, Q., Panksepp, J. B. & Lahvis, G. P. Empathy Is Moderated by Genetic Background in Mice. *PLoS ONE*. **4**, e4387 (2009).
55. Ito, W., Erisir, A. & Morozov, A. Observation of Distressed Conspecific as a Model of Emotional Trauma Generates Silent Synapses in the Prefrontal-Amygdala Pathway and Enhances Fear Learning, but Ketamine Abolishes those Effects. *Neuropsychopharmacology* **40**, 2536–2545 (2015).
56. Silverstein, S. E. et al. A distinct cortical code for socially learned threat. *Nature* **626**, 1066–1072 (2024).

57. Martínez-Rivera, F. J. et al. Prolonged avoidance training exacerbates OCD-like behaviors in a rodent model. *Transl Psychiatry*. **10**, 212 (2020).
58. Diehl, M. M., Moscarello, J. M. & Trask, S. Behavioral outputs and overlapping circuits between conditional fear and active avoidance. *Neurobiol. Learn. Mem.* **213**, 107943 (2024).
59. Packheiser, J. et al. Audible pain squeaks can mediate emotional contagion across pre-exposed rats with a potential effect of auto-conditioning. *Commun. Biol.* **6**, 1085 (2023).
60. Parsana, A. J., Moran, E. E. & Brown, T. H. Rats learn to freeze to 22-kHz ultrasonic vocalizations through autoconditioning. *Behav. Brain Res.* **232**, 395–399 (2012).
61. Pereira, A. G., Cruz, A., Lima, S. Q. & Moita, M. A. Silence resulting from the cessation of movement signals danger. *Curr. Biol.* **22**, R627–R628 (2012).
62. Fanselow, M. S. & LeDoux, J. E. Why We Think Plasticity Underlying Pavlovian Fear Conditioning Occurs in the Basolateral Amygdala. *Neuron* **23**, 229–232 (1999).
63. Maren, S. What the Amygdala Does and Doesn't Do in Aversive Learning: Fig. 1. *Learn. Mem.* **10**, 306–308 (2003).
64. Wassum, K. M. Amygdala-cortical collaboration in reward learning and decision making. *eLife* 11, e80926 (2022).
65. McReynolds, J. R., Christianson, J. P., Blacktop, J. M. & Mantsch, J. R. What does the Fos say? Using Fos-based approaches to understand the contribution of stress to substance use disorders. *Neurobiol. Stress.* **9**, 271–285 (2018).
66. McEwen, B. S., Nasca, C. & Gray, J. D. Stress Effects on Neuronal Structure: Hippocampus, Amygdala, and Prefrontal Cortex. *Neuropsychopharmacol. Off Publ Am. Coll. Neuropsychopharmacol.* **41**, 3–23 (2016).
67. Atrooz, F., Alkadhi, K. A. & Salim, S. Understanding stress: Insights from rodent models. *Curr. Res. Neurobiol.* **2**, 100013 (2021).
68. Piantadosi, P. T., Yeates, D. C. M. & Floresco, S. B. Cooperative and dissociable involvement of the nucleus accumbens core and shell in the promotion and inhibition of actions during active and inhibitory avoidance. *Neuropharmacology* **138**, 57–71 (2018).
69. Roughley, S. & Killcross, S. The role of the infralimbic cortex in decision making processes. *Curr. Opin. Behav. Sci.* **41**, 138–143 (2021).
70. Gonzalez, S. T. & Fanselow, M. S. The role of the ventromedial prefrontal cortex and context in regulating fear learning and extinction. *Psychol. Neurosci.* **13**, 459–472 (2020).
71. Barker, J. M., Taylor, J. R. & Chandler, L. J. A unifying model of the role of the infralimbic cortex in extinction and habits. *Learn. Mem.* **21**, 441–448 (2014).
72. Mukherjee, A. & Caroni, P. Infralimbic cortex is required for learning alternatives to prelimbic promoted associations through reciprocal connectivity. *Nat. Commun.* **9**, 2727 (2018).

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Author contributions

S.R. designed the study, wrote the main manuscript text, conducted statistical analyses, and prepared figures. S.R., C.K., L.W., and H.D. conducted behavioral and immunohistochemical experiments. S.R. and M.M.D. secured funding for the experiments in this study. M.M.D. supervised this study. All authors reviewed and revised the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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