Sex Differences in Central Amygdala Reactivity following psychedelic drug exposure

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Author Contributions

D.W.E. contributed to overall conception, experimental design, surgical procedures, behavioral experiments, data analysis, video processing, histology, and manuscript drafting. S.G.Q. contributed to behavioral experiments, microscopy, histology, and manuscript editing. M.C.R. contributed to behavioral video scoring and histology. M.C.R. contributed to histology. M.A.H. contributed to overall conception, experimental design, behavioral experiments, and manuscript editing.

ABSTRACT

Psilocybin, and its active metabolite psilocin, have been shown to elicit rapid and long-lasting symptom improvements in a variety of affective psychiatric illnesses. However, the specific mechanisms behind these therapeutic effects remain relatively unknown. The central amygdala (CeA) is a primary output region within the extended amygdala that is heavily dysregulated in affective psychiatric disorders. Here, we utilized fiber photometry to measure changes in CeA reactivity to an aversive air puff stimulus after psilocin administration, both acutely and at varying prolonged time points. We found that administration of psilocin increased reactivity acutely in females, but not males. Additionally, we show that one dose of psilocin produced decreases in reactivity in males, but not females, 28-days post administration, with decreases seen as early as 2-days post administration. We further report stimulus-specific changes in CeA reactivity after psilocin with differential responsivity to an aversive air puff stimulus and an auditory stimulus. We also measured changes in behavioral response to the air puff stimulus and report sex differences in threat responding but not exploratory behavior or general locomotion. This study provides evidence that a single dose of psilocin can elicit sex-specific, dynamic, and enduring changes in CeA reactivity to an aversive stimulus. These data are an important step towards dissecting circuit-based mechanisms underlying the effects of psychedelics.

INTRODUCTION

In 2018, psilocybin, the hallucinogenic compound found in *psilocybe cubensis* mushrooms, was granted breakthrough therapy designation by the Food and Drug Administration as a potential therapeutic for patients with treatment-resistant depression (TRD). Numerous clinical studies have shown that psilocybin, and its active metabolite psilocin [1-3], can induce rapid, long-lasting improvements in several psychiatric diagnoses including depression, anxiety, and substance use disorder [4-12]. These effects seem to be long lasting, as studies have shown sustained improvements in symptoms that are seen as long as 4 years following administration [13]. Research on psychedelic compounds thus far has focused primarily on clinical fMRI human imaging studies, and receptor pharmacology. Human fMRI studies have shown that psychedelics produce a robust alteration in network connectivity [8, 14-19]. Receptor pharmacology studies have illustrated the ability of these compounds to promote synaptogenesis [20-22] and identified the serotonin (5-HT) $_{2A}$ receptor to be instrumental in mediating the actions of psychedelic compounds such as psilocybin and lysergic acid diethylamide (LSD) [16, 23-28]. Despite the observed clinical outcomes in psychedelic studies, the region-specific mechanisms behind these therapeutic effects remain relatively unknown.

The central amygdala (CeA) is a primary output region within the extended amygdala that receives input regarding internal and external arousal state and coordinates appropriate behavioral responses [29, 30]. The CeA has also been shown to be heavily implicated in anxiety, depression, and PTSD [29, 31-36]. Preclinical studies have shown that administration of the 5-HT_{2AR} agonist psychedelic compounds LSD and 2,5-dimethoxy-4-iodoamphetamine (DOI) both promote increases in Fos expression in the amygdala [37, 38] and that systemic and local DOI administration in the amygdala promotes suppression of fear response [38]. Several clinical studies have shown psilocybin can produce alterations in reactivity and connectivity of the amygdala that correlate with a positive therapeutic outcome [12, 15, 17, 39-41]. A recent clinical imaging study showed that amygdala reactivity was significantly decreased 1-week following psilocybin administration, with lasting changes in affect seen at a one-month follow up [41]. To better understand the mechanism behind these changes, preclinical models provide a more targeted approach to examine brain region-specific activity.

While the literature for psychedelic research is fairly robust, there remains a large gap in our knowledge and understanding of how these chemicals elicit their therapeutic effects. Addressing this area of questioning has never been more pertinent given the numerous clinical trials conducted and currently underway. Answering these questions will provide evidence and information towards a better understanding of these drugs that could then lend to the development of novel treatment options. Additionally, given the potential ability of these drugs to treat various psychiatric illnesses, understanding the region- and circuit-based mechanisms behind their effects could uncover shared alterations in brain function between these different conditions. The current study utilized a preclinical approach to determine how the psychedelic compound, psilocin, altered CeA reactivity to an aversive stimulus in male and female Sprague Dawley rats. Utilizing fiber photometry in conjunction with an aversive air puff stimulus, we were able to demonstrate a robust and reliable CeA reactivity response. We then used this model to determine how administration of psilocin altered reactivity of the CeA during acute exposure and during more prolonged timepoints. Here, we show that there are sex differences in the effects of psilocin on CeA reactivity. Additionally, we show that psilocin elicited long-term decreases in CeA reactivity in males that were not seen in females or in the vehicle control. Utilizing an auditory only air puff stimulus, we show differential reactivity of the CeA suggesting that the observed changes were stimulus-specific and dynamic in nature. Sex-dependent behavioral responses to the air puff stimulus resulting from psilocin administration were also observed.

RESULTS

Acute Changes in CeA Reactivity in Response to an Aversive Air Puff Stimulus Following Psilocin Administration

At 8 weeks of age, male and female Sprague Dawley rats received bilateral injection of pGP-AAV-syn-jGCaMP7f-WPRE into the CeA immediately followed by installation of fiber optic cannulas above the injection site (Fig. 1A,1B). After allowing 3-weeks for virus incubation, animals underwent a series of fiber photometry experiments to test the effects of psilocin on fluctuations in calcium dynamics within the CeA following an aversive air puff stimulus (Fig. 1A). After connecting a fiber optic patch cord to their cannula, each animal was

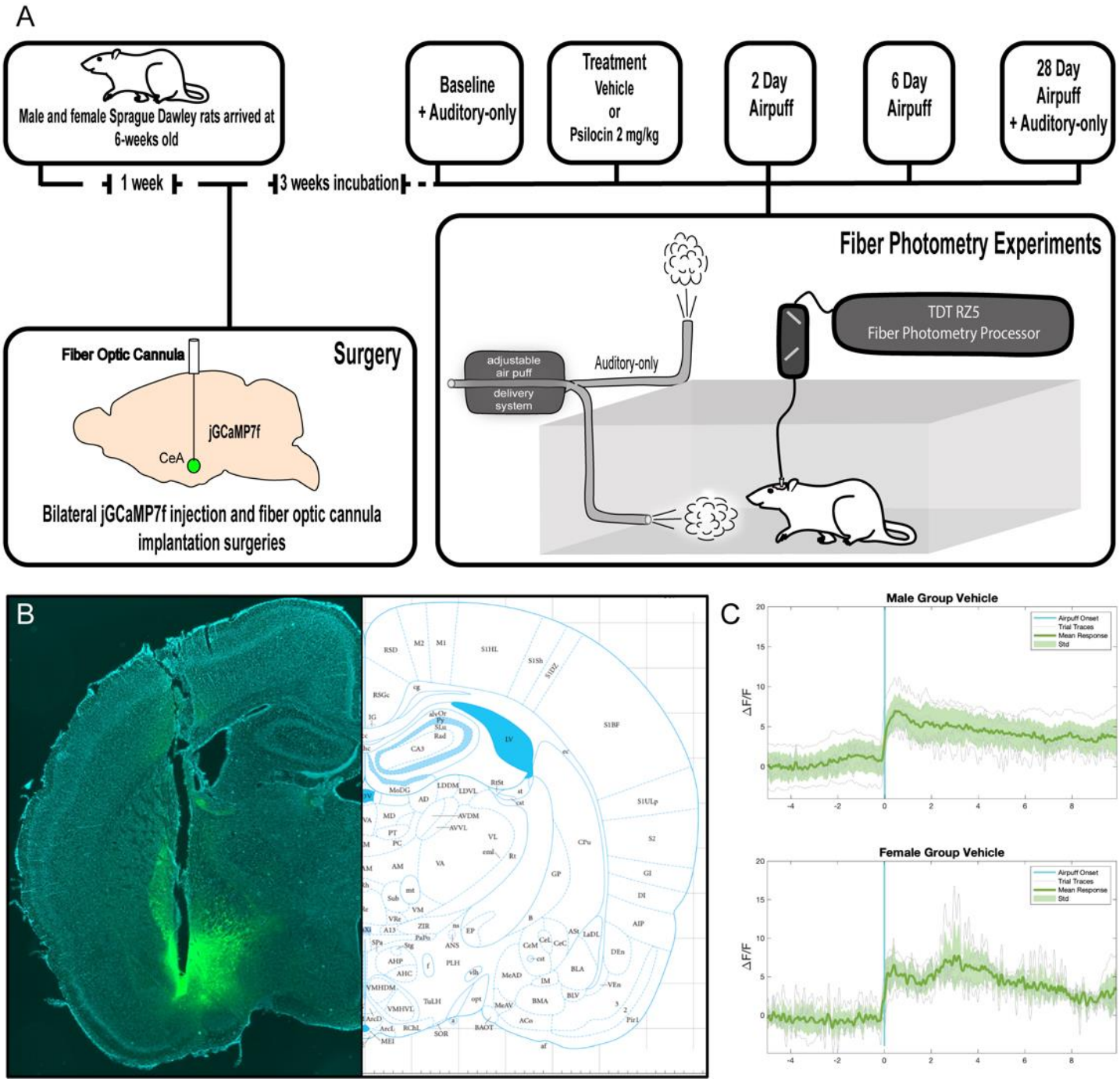


Figure 1. (**A**) Experimental timeline. Animals arrived at 6 weeks and allowed to habituate for 1-week prior to surgeries. Bilateral injection of calcium sensor (jGCaMP7f) and implantation of fiber optic cannulas were conducted. After 3 weeks for viral incubation, animals underwent a series of fiber photometry experiments. (**B**) Representative coronal section showing fiber placement and GCaMP expression. (**C**) Mean air puff traces showing consistency of CeA signal in response to the air puff stimulus.

given 10 minutes to habituate to an open field box before each recording session began. For each animal, two air puffs were given with an inter-trial interval (ITI) of 5 min and trace plots were generated showing change in fluorescence as a function of baseline (ΔF/F). Traces were collected 5 seconds before and 10 seconds following administration of the air-puff (Fig. 1C).

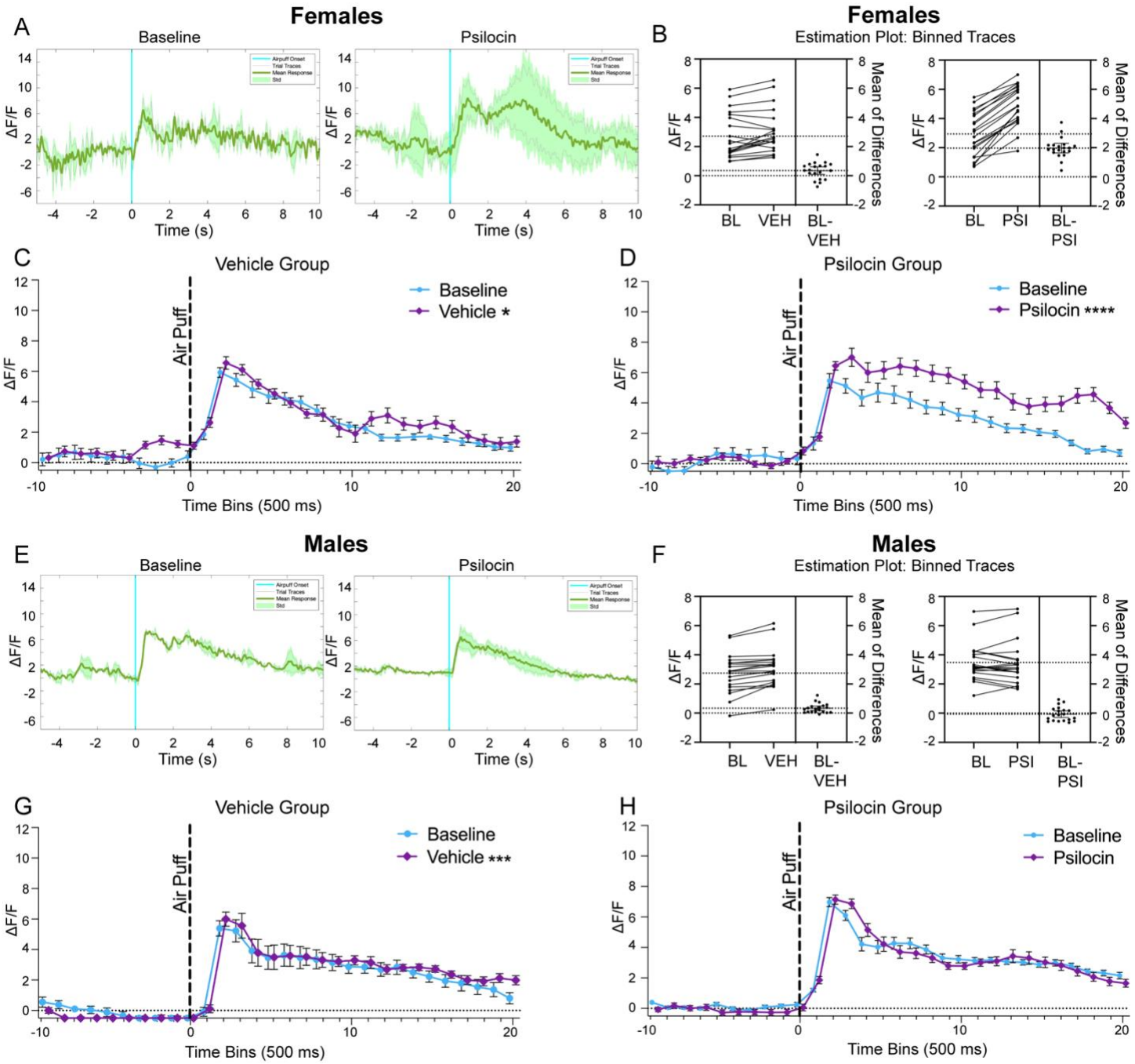


Figure 2. *Acute Changes in CeA Reactivity in Response to an Aversive Air Puff Stimulus Following Psilocin Administration* **(A)** Female psilocin group: representative raw ΔF/F traces showing an individual at baseline and while on psilocin. **(B)** Females: Estimation plots for binned trace plots comparing baseline to treatment. Each data point corresponds to a 500ms bin averaged across subjects +/- SEM. (**C)** Female vehicle group: air-puff trace plots of changes in CeA fluorescence in 500ms bins. Data points represent group averages within 500ms binned window +/- S.E.M. **(D)** Female psilocin group: air-puff trace plots of changes in CeA fluorescence in 500ms bins. Data points represent group averages within 500ms binned window +/- S.E.M. **(E)** Male psilocin group: representative raw ΔF/F traces showing an individual at baseline and while on psilocin. **(F)** Males: Estimation plots for binned trace plots comparing baseline to treatment. Each data point corresponds to a 500ms bin averaged across subjects +/- SEM. **(G)** Male vehicle group: air-puff trace plots of changes in CeA fluorescence in 500ms bins. Data points represent group averages within 500ms binned window +/- S.E.M. **(H)** Male psilocin group: air-puff trace plots of changes in CeA fluorescence in 500ms bins. Data points represent group averages within 500ms binned window +/- S.E.M.

To test the acute effects of psilocin on CeA reactivity, we utilized the fiber photometry air puff model described above to measure changes between baseline and 30 minutes post administration of either a vehicle or psilocin (2 mg/kg) subcutaneous injection. Following a 10-minute habituation period, air puffs were delivered as described above. Mean traces were taken from two separate air puff responses within a session (Fig. 2A, 2E). These traces were then collapsed into 500ms bins for statistical analysis (Fig. 2C, D, G, H). To compare baseline to treatment, paired t-tests were used comparing time bins following the air puff collapsed across subjects. In the female vehicle control group (n=9), a paired t-test showed a significant increase between baseline and treatment (p = 0.01, t (19) =2.780, mean of differences = 0.34; Fig. 2B, 2C). In the female psilocin group (n = 7), a paired t-test revealed a significant increase between baseline and treatment (p < 0.0001, t (19) =12.63, mean of differences = 1.97; Fig. 2B, 2D). Though changes were seen in both vehicle and psilocin, the increase in reactivity was much greater in the psilocin group compared to the vehicle control (Fig. 2B). In the male vehicle control group (n = 10), a paired t-test revealed a significant increase in reactivity between baseline and treatment (p = 0.0003, t (19) =4.452, mean of differences = 0.33; Fig. 2F, 2G). In the male psilocin group (n = 11), a paired t-test revealed no significant difference between baseline and treatment (p = 0.54, t (19) =0.6231, mean of differences = -0.06; Fig. 2F, 2H).

Prolonged Changes in CeA Reactivity in Response to an Aversive Air Puff Stimulus Following Psilocin Administration

Next, to test for any long-term alterations in CeA reactivity following a single dose of psilocin, fiber photometry recordings were collected 2-days, 6-days, and 28-days post administration of psilocin or a vehicle control. In the females, a 2-way analysis of variance (ANOVA) comparing area under curve (AUC) of the group averaged individual mean traces between baseline and the 28-day follow up revealed no difference between the vehicle and psilocin groups (Treatment x Time: F (1, 14) = 0.003383, p = 0.95; Treatment: F (1, 14) = 0.09635, p = 0.76; Time: F (1, 14) = 0.2126, p = 0.65; Fig. 3B). Additionally, no differences were found between the vehicle and psilocin at the follow up timepoints (Treatment x Time: F (2, 28) = 1.437, p = 0.25; Treatment: F (1, 14) = 0.0003490, p = 0.99; Time: F (2, 28) = 3.067, p = 0.06; Fig. 3B). However, in the vehicle control females, a

repeated measures 1-way ANOVA of the binned trace plots following air puff comparing baseline to treatment, 2-, 6-, and 28-day follow up revealed a significant effect of treatment ($F(3, 57) = 50.02$, $p < 0.0001$; Fig. 3C). Dunnett's post hoc analysis revealed a significant increase in reactivity at the 2-day ($p < 0.0001$) and the 6-day follow up ($p < 0.0001$) compared to baseline. In the female psilocin group (Fig. 3A, 3D), a repeated measures 1-way ANOVA of the binned trace plots following air puff comparing baseline to the treatment, 2-, 6-, and 28-day follow up time points revealed a significant effect of treatment ($F(3, 57) = 54.13$, $p < 0.0001$; Fig. 3D). Dunnett's post hoc analysis revealed significant increases in reactivity at the 2-day ($p < 0.0001$) and 6-day follow up ($p < 0.0001$) compared to baseline.

In the males, a 2-way ANOVA comparing AUC of the group averaged individual mean traces revealed a significant treatment x time interaction between the vehicle and psilocin group from the baseline to the 28-day follow up (Treatment x Time: $F(1, 19) = 5.068$, $p = 0.03$; Treatment: $F(1, 19) = 0.2503$, $p = 0.62$; Time: $F(1, 19) = 1.361$, $p = 0.26$; Fig. 3F) with a significant reduction in reactivity at the 28-day follow up seen in the psilocin group ($p = 0.04$). An additional 2-way ANOVA comparing AUC for just the follow up days showed a main effect of treatment producing reductions in reactivity in the psilocin group compared to the vehicle control at the follow up days (Treatment x Time: $F(2, 38) = 0.2113$, $p = 0.81$; Treatment: $F(1, 19) = 4.926$, $p = 0.03$; Time: $F(2, 38) = 0.7906$, $p = 0.46$; Fig. 3F). In the male vehicle control group, a repeated measures 1-way ANOVA of the binned trace plots following air puff revealed a significant effect of treatment ($F(3, 57) = 5.554$, $p = 0.002$; Fig. 3G). Dunnett's post hoc analysis revealed a significant increase in reactivity at the 28-day follow up ($p = 0.01$) compared to baseline. In the male psilocin group (Fig. 3E, 3H), a repeated measures 1-way ANOVA of the binned trace plots following air puff revealed a significant effect of treatment ($F(3, 57) = 72.60$, $p < 0.0001$; Fig. 3H). Dunnett's post hoc analysis revealed a significant decrease in reactivity at the 2-day ($p < 0.0001$), 6-day ($p < 0.0001$), and 28-day follow up ($p < 0.0001$) compared to baseline.

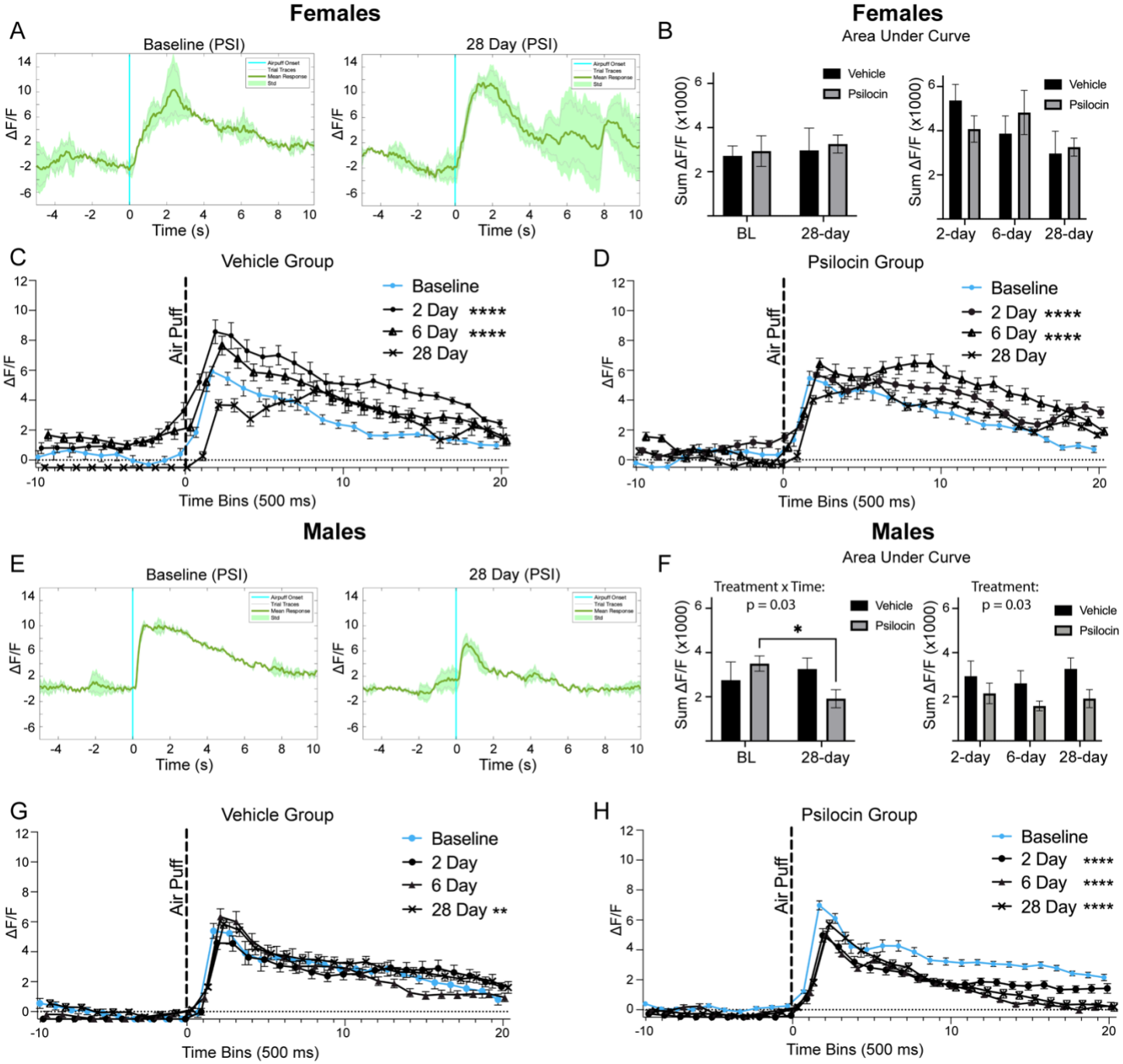


Figure 3. *Prolonged Changes in CeA Reactivity in Response to an Aversive Air Puff Stimulus Following Psilocin Administration* **(A)** Female psilocin group: representative raw ΔF/F traces showing an individual at baseline and at the 28-day follow up. **(B)** First graph: area under curve (AUC) histogram plots comparing baseline to the 28-day follow up. Second graph: changes in AUC at each of the follow up time points between the vehicle and psilocin group. Mean AUC +/- S.E.M. **(C)** Female vehicle group: air-puff trace plots of changes in CeA fluorescence in 500ms bins. Data points represent group averages within 500ms binned window +/- S.E.M. **(D)** Female psilocin group: air-puff trace plots of changes in CeA fluorescence in 500ms bins. Data points represent group averages within 500ms binned window +/- S.E.M. **(E)** Male psilocin group: representative raw ΔF/F traces showing an individual at baseline and while on psilocin. **(F)** First graph: AUC histogram plots comparing baseline to the 28-day follow up between male vehicle and psilocin groups. Second graph: changes in AUC at each of the follow up time points between the male vehicle and psilocin group. Mean AUC +/- S.E.M. **(G)** Male vehicle group: air-puff trace plots of changes in CeA fluorescence in 500ms bins. Data points represent group averages within 500ms binned window +/- S.E.M. **(H)** Male psilocin group: air-puff trace plots of changes in CeA fluorescence in 500ms bins. Data points represent group averages within 500ms binned window +/- S.E.M.

Acute and Prolonged Behavioral Effects of Psilocin

For all behavior measures, scoring took place during the ITI between the first and second air puff. To determine if psilocin elicited global behavioral changes, video recordings were scored to determine changes in locomotion between the different time points. In the females, a 2-way ANOVA found no differences between vehicle and psilocin groups (Treatment x Time: F (3, 42) = 0.2118, *p* = 0.89; Treatment: F (1, 14) = 2.238, *p* = 0.16; Time: F (2.497, 34.95) = 0.6065, *p* = 0.59; Fig. 4A). Additionally, in the males, a 2-way ANOVA found no differences in locomotion between vehicle and psilocin groups (Treatment x Time: F (3, 57) = 0.1054, *p* = 0.96; Treatment: F (1, 19) = 2.563, *p* = 0.12; Time: F (3, 57) = 1.255, *p* =0.29; Fig. 4B). Therefore, no significant alterations in locomotion were seen resulting from psilocin administration (Fig. 4A, 4B), although the females appeared to display increased locomotion as compared to males, regardless of treatment.

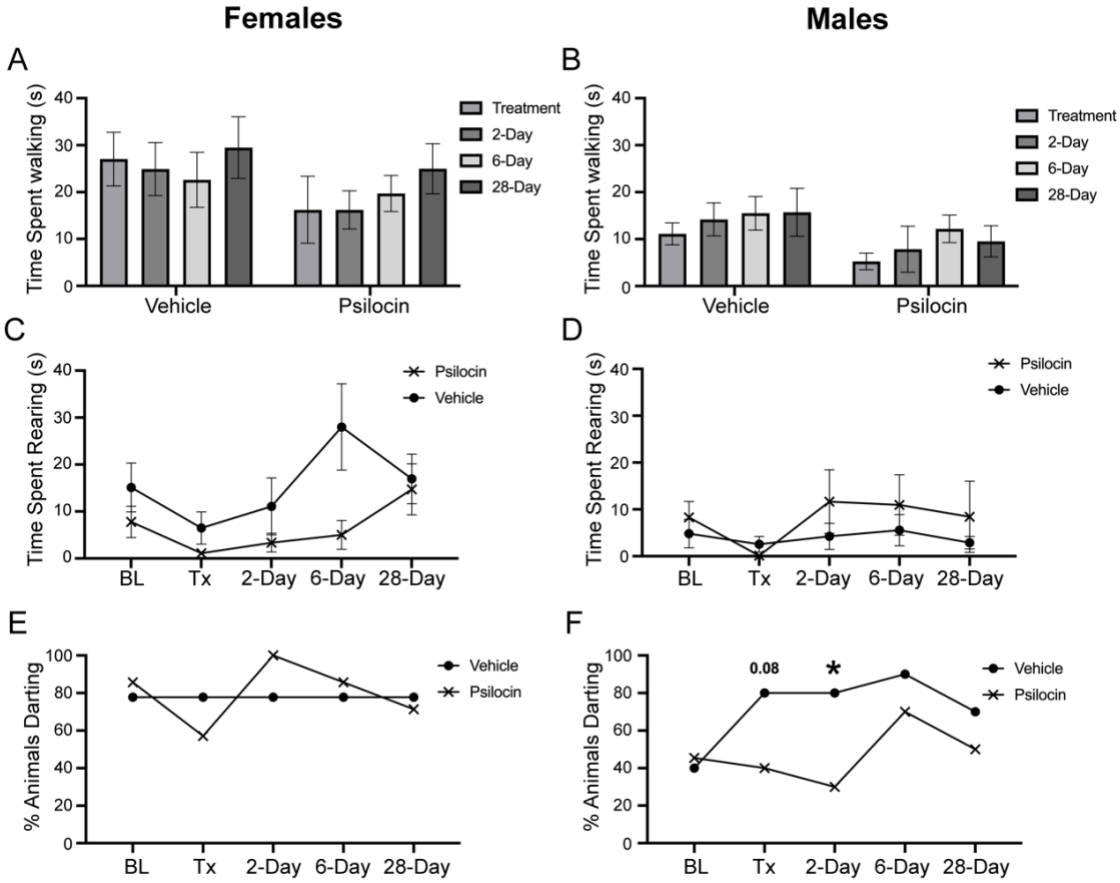


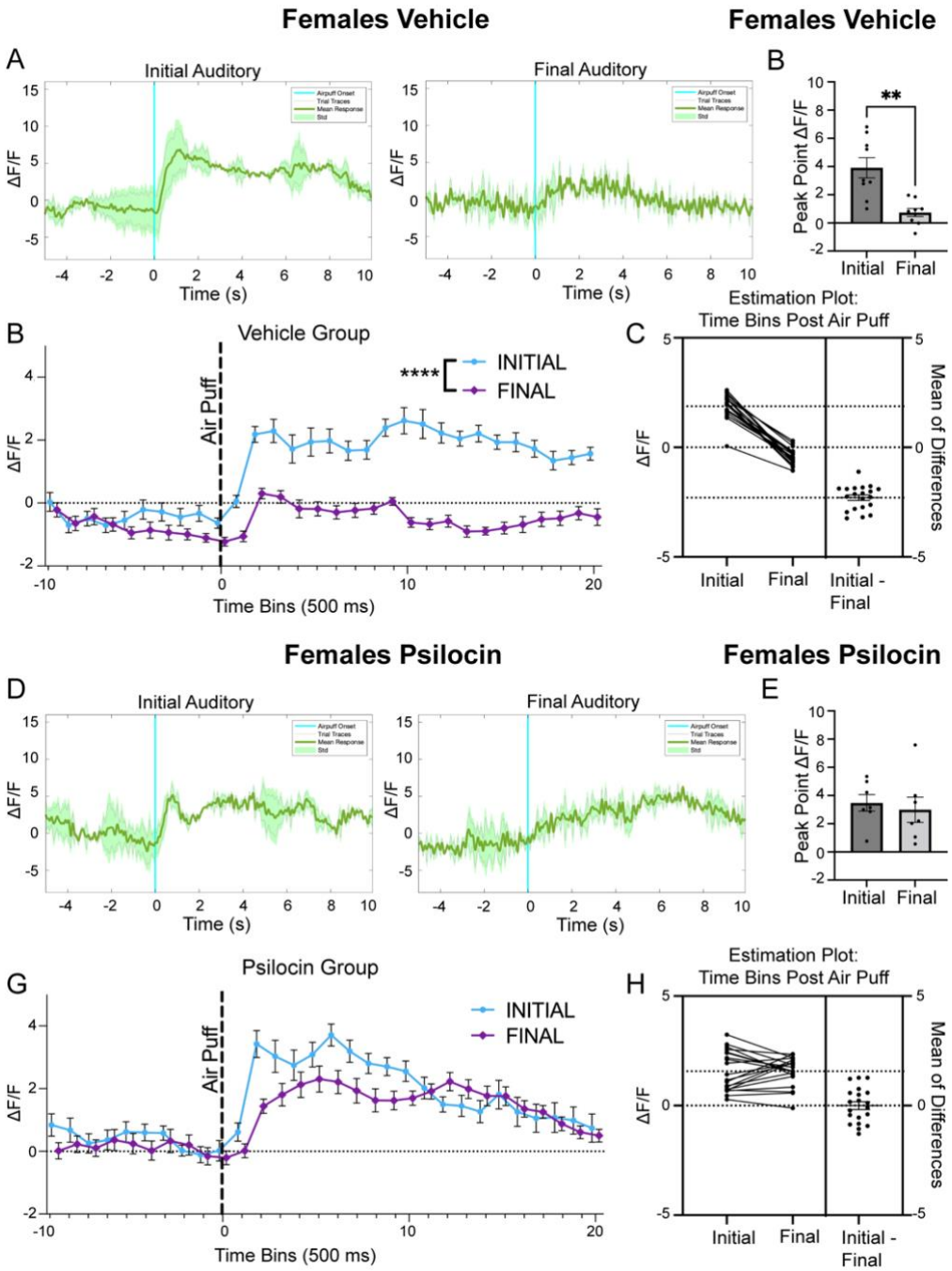
Figure 4. *Acute and Prolonged Behavioral Effects of Psilocin*. **(A)** Locomotion plots comparing female vehicle and psilocin groups at each time point. Data points are mean time in seconds +/- SEM. **(B)** Locomotion plots comparing male vehicle and psilocin groups at each time point. Data points are mean time in seconds +/- SEM. **(C)** Time spent rearing comparing female vehicle and psilocin groups at each time point. Data points are mean time in seconds +/- SEM. **(D)** Time spent rearing comparing male vehicle and psilocin groups at each time point. Data points are mean time in seconds +/- SEM. **(E)** Percentage of female subjects that darted immediately following air puff. **(F)** Percentage of male subjects that darted immediately following

To determine if psilocin impacted exploratory behavior, behavioral videos were scored for time spent rearing. In the females, a 2-way ANOVA revealed no differences between the vehicle and psilocin group, however there was a strong trend for both main effects, where psilocin females showed reductions in time spent rearing as compared to vehicle (Treatment x Time: $F(4, 56) = 1.345$, $p = 0.26$; Treatment: $F(1, 14) = 4.433$, $p = 0.05$ Time: $F(4, 56) = 2.486$, $p = 0.05$; Fig. 4C). In the males, a 2-way ANOVA revealed no differences between the vehicle and psilocin group (Treatment x Time: $F(4, 76) = 0.5546$, $p = 0.70$; Treatment: $F(1, 19) = 0.7576$, $p = 0.39$; Time: $F(4, 76) = 1.190$, $p = 0.32$; Fig. 4D).

Next, we were interested in the acute behavioral response to the air puff stimulus and how treatment might alter this response. To probe potential differences in threat responding to the air puff stimulus, we looked at darting behavior following the first air puff. Darting would be defined as the immediate flee upon receiving an air puff. In the females, no differences were seen in the percentage of animals that darted between groups in any of the time points (Fig. 4E). However, there did appear to be a slight trend for a decrease in darting in the psilocin group while on psilocin ($p = 0.59$ by Fisher's exact test) and an increase in darting at the 2- day follow up air puff recording ($p = 0.48$ by Fisher's exact test; Fig. 4E). In contrast, the male psilocin group showed a strong trending decrease in darting seen between baseline and psilocin ($p = 0.08$ by Fisher's exact test, Fig. 4F) with a significant decrease in darting seen at the 2-day follow up ($p = 0.03$ by Fisher's exact test, Fig. 4F). Interestingly, though differences were not significant at every time point, psilocin did seem to prevent a trending increase in darting behavior seen in the male vehicle control group (Fig.4F).

Changes in CeA Reactivity in Response to an Auditory Stimulus Following Psilocin Administration

To test whether the animals developed a conditioned response to the tone associated with the air-puff stimulus, and whether psilocin treatment altered the response to the auditory stimulus alone, a separate auditory-only session was conducted that followed the same parameters as the air puff with the exceptions being that the ITI was reduced to 3 minutes and the air puff apparatus was facing outside of the box to maintain the same auditory tone without the physical sensation of the air puff. An initial auditory-only session

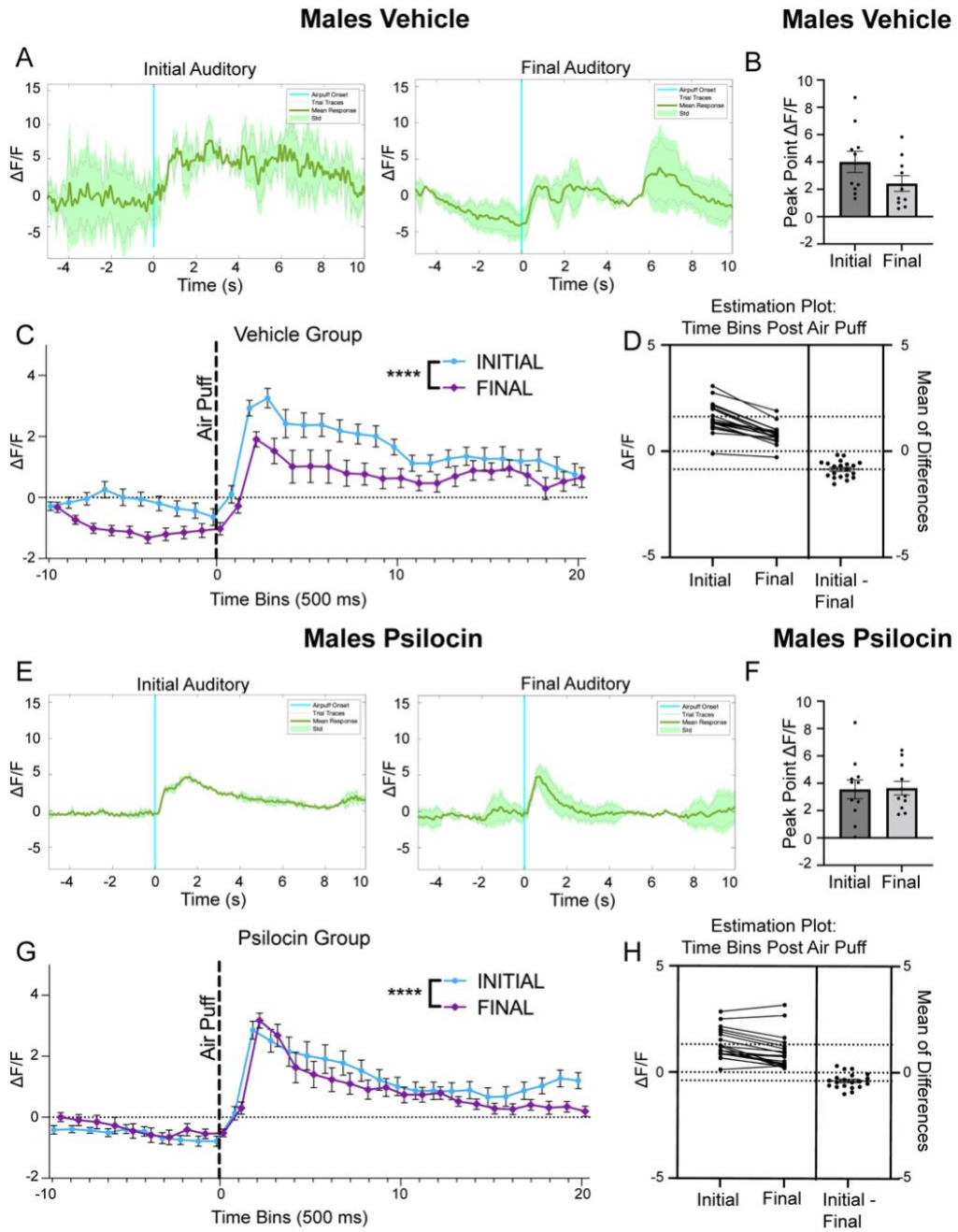


occurred following baseline recordings, and then a final auditory-only session occurred following the 28-day recording.

In the vehicle control females (Fig. 5A), a paired two-tailed t-test comparing the peak point of binned mean traces found a significant decrease in reactivity from the initial to the final auditory condition (p = 0.004, t (8) =3.934, mean of differences = -3.17; Fig. 5B). An additional paired t-test comparing time bins following air puff for the vehicle group found a significant decrease from the initial auditory to the final auditory condition (p < 0.0001, t (19) =18.28, mean of differences = -

Figure 5. *Changes in CeA Reactivity in Response to an Auditory Stimulus Following Psilocin Administration in Female Subjects.* **(A)** Female vehicle group: representative mean ΔF/F traces showing an individual at initial auditory compared to final auditory. **(B)** Female vehicle group: peak point of binned mean ΔF/F traces compared between initial and final auditory recordings. Data points represent each individual peak ΔF/F value +/- SEM. **(C)** Female vehicle group: air-puff trace plots of changes in CeA fluorescence in 500ms bins. Data points represent group averages within 500ms binned window +/- S.E.M. **(D)** Female vehicle group: Estimation plots for binned trace plots comparing baseline to treatment. Each data point corresponds to a 500ms bin averaged across subjects +/- SEM. **(E)** Female psilocin group: representative mean ΔF/F traces showing an individual at initial auditory compared to final auditory. **(F)** Female psilocin group: peak point of binned mean ΔF/F traces compared between initial and final auditory recordings. Data points represent individual peak ΔF/F value +/- SEM. **(G)** Female psilocin group: air-puff trace plots of changes in CeA fluorescence in 500ms bins. Data points represent group averages within 500ms binned window +/- S.E.M. **(H)** Female psilocin group: Estimation plots for binned trace plots comparing baseline to treatment. Each data point corresponds to a 500ms bin averaged across subjects +/- SEM.

2.30; Fig. 5B, 5C). In the psilocin female group (Fig. 5D), no significant differences were found between the initial and final auditory session in both a paired t-test of the peak point (p = 0.67, t (6) =0.4346, mean of differences = -0.49; Fig. 5E) as well as a paired t-test comparing the time bins following the air puff across subjects (p = 0.98, t (19) =0.01624, mean of differences = 0.002; Fig. 5G, 5H).



In the vehicle control males (Fig. 6A), a paired two-tailed t-test comparing peak point between initial and final auditory was not significant, though there was a strong trend towards a reduction at

Figure 6. *Changes in CeA Reactivity in Response to an Auditory Stimulus Following Psilocin Administration in Male Subjects.* **(A)** Male vehicle group: representative mean ΔF/F traces showing an individual at initial auditory compared to final auditory. **(B)** Male vehicle group: peak point of binned mean ΔF/F traces compared between initial and final auditory recordings. Data points represent each individual peak ΔF/F value +/- SEM. **(C)** Male vehicle group: air-puff trace plots of changes in CeA fluorescence in 500ms bins. Data points represent group averages within 500ms binned window +/- S.E.M. **(D)** Male vehicle group: Estimation plots for binned trace plots comparing baseline to treatment. Each data point corresponds to a 500ms bin averaged across subjects +/- SEM. **(E)** Male psilocin group: representative mean ΔF/F traces showing an individual at initial auditory compared to final auditory. **(F)** Male psilocin group: peak point of binned mean ΔF/F traces compared between initial and final auditory recordings. Data points represent individual peak ΔF/F value +/- SEM. **(G)** Male psilocin group: air-puff trace plots of changes in CeA fluorescence in 500ms bins. Data points represent group averages within 500ms binned window +/- S.E.M. **(H)** Male psilocin group: Estimation plots for binned trace plots comparing baseline to treatment. Each data point corresponds to a 500ms bin averaged across subjects +/- SEM.

the final auditory session (p = 0.06, t (9) =2.136, mean of differences = -1.58; Fig. 6B). An additional paired t-test comparing the time bins following the air puff of the initial and final auditory sessions found a significant decrease at the final auditory session compared to the initial (p < 0.0001, t (19) =9.719, mean of differences = -0.85; Fig. 6C, 6D). In the psilocin-treated male group (Fig. 6E), a paired two-tailed t-test comparing peak point from the initial to the final auditory session found no decrease (p = 0.92, t (10) =0.09932, mean of differences = 0.09; Fig. 6F). However, a paired t-test comparing entire binned trace plots found a significant decrease between the initial auditory and final auditory condition (p < 0.0001, t (19) =4.943, mean of differences = -0.39; Fig. 6G, 6H). While there was a decrease in both the vehicle and psilocin groups, there seemed to be a greater decrease in peak point in the vehicle group (Fig. 6B, 6F) and the estimation plots showed a much greater reduction in the vehicle (mean of difference = -0.85, Fig. 6D) as compared to the psilocin group (mean of difference = -0.39, Fig. 6H).

DISCUSSION

The present study explored the effects of the psychedelic compound, psilocin, on CeA reactivity to an aversive air puff stimulus using *in-vivo* fiber photometry recordings measuring changes in calcium dynamics during acute drug exposure and at 2- , 6- , and 28-days post drug administration. The study was designed to test multiple dimensions of the neurological and behavioral effects of psilocin by looking at the acute effects while on the drug, testing for long-term alterations in activity following drug administration, and by probing for potential sex differences in the effects of this drug. To our knowledge, this study is the first to utilize *in-vivo* fiber photometry to track region-specific changes and sex differences in neural activity both acute and long-term following the administration of a psychedelic compound. This approach provided a real-time analysis of fluctuations in calcium dynamics in the central amygdala (CeA) in response to an aversive air puff stimulus. We found there to be alterations in CeA reactivity in response to psilocin administration that differed between sexes and at different time points. In females, psilocin produced an exaggerated increase in CeA reactivity as compared to vehicle control (Fig. 2D). Interestingly, both vehicle and psilocin groups showed increases in reactivity at the 2- and 6-day follow up compared to baseline. In contrast, the male psilocin group did not show the same increase in reactivity seen in the females (Fig. 2H). Additionally, there was a decrease in CeA

reactivity at the 2-day, 6-day, and 28-day follow up (Fig. 3F, 3H) as compared to baseline. Vehicle control males showed no decrease in reactivity, with only a slight increase in reactivity seen at the 28-day follow up (Fig. 3G).

Given the various clinical studies illustrating the ability of psilocybin to produce alterations in amygdala reactivity and connectivity [8, 12, 15, 17, 39-41], we utilized a preclinical approach to determine whether the actions of psychedelic compounds on amygdala activity could be reproduced in a rodent model. Given that all but one of these studies showed a reduction in amygdala reactivity or connectivity that correlated with therapeutic improvements [8, 15, 17, 39-41], we hypothesized that psilocin would produce a similar decrease in reactivity. In alignment with these clinical findings, this study demonstrated that a single dose of psilocin, the psychoactive metabolite of psilocybin, produced persistent reductions in CeA reactivity to an aversive stimulus in male but not female rats. These findings lend to recent studies in both clinical [11, 13, 42] and preclinical [43] literature showing persistent reductions in depressive behavior after a single dose of psilocybin. Although the mechanism behind these therapeutic effects remains unknown, this study provides evidence towards a region-specific alteration involving the CeA that may play a role in the observed therapeutic effects.

This study revealed sex-specific effects of a psychedelic on reactivity within the CeA. In the female psilocin group, we observed an acute increase in reactivity to the air puff stimulus while on the drug that was not seen in the males. Additionally, we saw increases in reactivity at the 2- and 6-day follow up recording days, while we saw dramatic reductions in reactivity in the male subjects at each of the follow up recording days (Fig. 3B, 3D, 3E, 3G). A recent study looking at cerebral blood flow and the expression of the serotonin transporter (5-HTT) showed that, in response to an aversive predator odor, there were increases in blood flow and expression of the immediate early gene *c-Fos* in the CeA that were related to 5-HTT expression in males but not females [44]. These findings suggest potential sex-specific expression of serotonin receptors within the amygdala. Given the affinity of psilocin for the serotonin system, differential expression of serotonin receptors within the CeA or surrounding nuclei could explain the sex-specific effects seen in this study. Additionally, a previous study showed sex-specific differences in 5-HT_{2AR} expression at baseline, wherein females had lower expression in the orbitofrontal cortex than males [45]. Another potential explanation for the differential effects of

psilocin between males and females may be due to alterations in innervating regions to the CeA. For example, the OFC has been shown, in non-human primates as well as rodents, to have connections directly to the central amygdala as well as to the intercalated nuclei, a population of local inhibitory GABAergic neurons within the amygdala complex [46, 47]. Lack of activation of this circuit due to a reduced expression of OFC 5-HT_{2AR} in females could prevent an inhibition of CeA firing, thus causing the increase in reactivity seen while on psilocin in females but not males. Future experiments would be needed to examine the effects of psilocin onto CeA innervating brain regions.

To determine the effect of psilocin administration on associative learning, we utilized an auditory-only stimulus recording taken after the initial baseline recordings and then again after the 28-day recording. Interestingly, in both males and females we found that the vehicle control group showed a strong desensitization in CeA response to the final auditory-only stimulus compared to the initial recording. In the psilocin group, the CeA reactivity was not reduced at the final auditory in the females, while in the males we did see a reduction though to a lesser degree than the vehicle control group (Fig. 6). Given the reductions in reactivity seen in the standard air puff stimulus, the sustained reactivity to the auditory stimulus in the psilocin group suggests that the decreases in reactivity we found under the air-puff conditions were not due to an overall blunting of CeA function. Instead, these data suggest a more dynamic alteration in CeA reactivity in response to different types of stimuli. One potential interpretation of these data is that psilocin may influence associative learning to an aversive stimulus. Several studies have shown that 5-HT_{2A} agonist psychedelics produce improvements in various dimensions of cognition, including associative learning [48-50]. Another potential interpretation of these findings could be that psilocin created a more robust/meaningful memory of the air puff. Many of the clinical studies cite participants rating their experience during a psilocybin treatment as one of the most meaningful experiences of their life [13, 51, 52]. Additionally, increases in plasticity could contribute to differences in memory reconsolidation during air puff administration while on the drug.

In summary, our findings provide evidence that administration of the psychedelic compound, psilocin, produces dynamic, sex-specific changes in the CeA, a key nucleus within the amygdala complex. We have also shown that there are sex differences in CeA reactivity while on psilocin. Additionally, we show that a single

administration of psilocin produced persistent changes in CeA reactivity that are seen as early as 2 and as long as 28 days following administration of the drug in males, but not females. Given that dysregulation of the amygdala is a hallmark in many different psychiatric disorders, including anxiety, MDD, and PTSD [29, 31-36] and that psychedelic compounds show promise in treating many of these illnesses [4, 6-11], these findings provide important information to a region specific alteration that may play a part in the therapeutic effects seen in the clinical literature. Additionally, the long-term reductions in amygdala reactivity seen with the air puff stimulus do not seem to be the result of overall desensitization of the CeA, given the sustained response in the auditory-only stimulus response seen in the psilocin group for both males and females. Future work will be required to explore potential circuit-based mediators contributing to these observed alterations in CeA reactivity, but currently these findings provide important evidence for a targeted region-specific alteration underlying the potential therapeutic effects of psilocin and psilocybin.

METHODS

Animals/Stereotaxic Surgery

Male and female Sprague Dawley rats (Envigo, Indianapolis, IN) were housed in a humidity- and temperature-controlled (22°C) vivarium on a regular 12-h light/dark cycle. Rats were ordered to arrive at 6-weeks old and were group-housed and had ad libitum access to food and water. Following intracranial surgery, animals were single housed to avoid potential damage to the cannulas from cage mates. Animals were stored in cages with flat lids, had rear access to water, and were given food in bowls to avoid damage to implants. Adult male and female rats, (200-400 g), were used for all experiments. Bilateral infusions of pGP-AAV-syn-jGCaMP7f-WPRE (Addgene plasmid# 104488-AAV9) were injected at a rate of 100nl/minute into the CeA at coordinates (AP: -2.0mm; ML: +/- 3.9mm; DV: -8.0mm). Directly following injection, Doric 200um NA 0.37 silica optic fiber cannulas with a 9mm tip were inserted at DV -7.7mm. Metabond (Parkell, Brentwood, NY) was used to secure cannulas in place and act as a headcap.

Drug Administration

On the day of treatment, animals received a subcutaneous (s.c.) injection of either vehicle (0.9% Saline with 2% glacial acetic acid) or a 2 mg/kg psilocin (Cayman Chemical, graciously provided by Dr. Bryan Roth) dissolved in 2% glacial acetic acid, as previously described [53]. Psilocin solutions were prepared fresh on the day of testing and were stored along with vehicle solution at 4ºC for 30 minutes prior to first injection. Injections were given at a 1mg/ml volume 30 minutes prior to the beginning of fiber photometry recordings.

Fiber Photometry Recording

For this study, the calcium sensor jGCaMP7f was used to measure neuronal calcium dynamics in response to a stimulus as a measure of reactivity. Animals were allowed to habituate in the behavioral room for 1 hour prior to the beginning of a recording session. During recording, rats were placed in red-light sound attenuated behavioral cabinets and allowed to acclimate in an open field box for a period of 10 min. Ca^{2+} signals from subjects, with 200µm optic fibers placed .3mm above the injection site in the CeA, were recorded using a TDT RZ5 real time processor equipped with the TDT Synapse program (Tucker-Davis Technologies, Alachua, FL). For each recording, jGCaMP7f was excited using both a 465nm calcium-dependent signal and a 405nm isosbestic control. MATLAB script [54] was then used to analyze raw signal. Changes in fluorescence as a function of baseline (ΔF/F) were calculated through smoothing isosbestic control channel signal , scaling the isosbestic signal by regressing it onto smoothed GCaMP signal, and then generating a predicted 405nm from the regression to remove calcium independent signaling from the raw GCaMP signal to control for artifact from movement, photo-bleaching, and fiber bending artifacts [55]. Peri-event plots were then generated by averaging changes in fluorescence (ΔF/F) across two different air-puff trials to generate a mean change in reactivity [54]. All subjects were video recorded during stimulus presentations and behavior was scored using the Behavioral Observation Research Interactive Software (BORIS) program [56]. Behavioral analysis was performed by an experimenter blind to the experimental status of each animal.

Air Puff Stimulus

An aversive air puff stimulus was designed using tubing connected to the air supply faucet in the behavior room. Air pressure was set at 85 psi and air flow was controlled using a Parker solenoid (Part #003-0868-900) powered by a custom made 12V electrical circuit box set to open for a 500ms window. This solenoid circuit was connected through a BNC port on the TDT RZ5 system to allow for opening of the solenoid and creation of a simultaneous timestamp through the Synapse program. Before the beginning of each recording day, animals were allowed to habituate to the behavior room for 1 hour in their home cage. Each session consisted of a 10-minute habituation period before beginning a recording session that consisted of administration of two air-puffs with a 5-minute ITI between them.

Perfusion/Tissue Collection

Rats were transcardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (w/*v*). Brains were extracted, stored in 4% paraformaldehyde for 24h, then transferred to 30% sucrose (*w/v*) and stored at 4ºC until sectioning. Using a freezing microtome, tissue was sliced into 40µm thick sections and stored in cryoprotectant (30% *v/v* ethylene glycol + 30% *w/v* sucrose in phosphate buffered saline) at 4ºC until use.

Immunofluorescence Staining

6 slices ranging from coordinates -1.75 to -2.75 from bregma were chosen from each animal to amplify GFP signal. Samples were washed 3 times in phosphate buffered saline (PBS) prior to being blocked for 1h in 5% normal donkey serum + 0.3% Triton X in PBS. Samples were then incubated in chicken anti-GFP (Abcam 13970; 1:1000) in 0.5% normal donkey serum + 0.3% Triton X in PBS overnight at 4ºC. The next day, samples were washed three times with PBS and then were incubated in secondary solution containing Samples were incubated in blocking solution for 1 hr followed by primary solution overnight at room temperature and secondary solution containing goat anti-chicken 488 (Jackson 703-545-155; 1:700) in 0.5% normal donkey serum + 0.3% Triton X in PBS for 2 hr at room temperature. Following 3 additional PBS washes, tissue slices were mounted and coverslipped with Vectashield ® HardSet™ Antifade Mounting Medium with DAPI (H1500,

Vector Laboratories, Burlingame, CA) and stored at 4ºC until imaging with the Keyence BZ-X800 fluorescence microscope.

Statistical Analysis

Raw data from the TDT RZ5 machine were initially analyzed using MATLAB code [54] for raw data import, artifact removal, down sampling, and detrending. Changes in fluorescence ($\Delta F/F$) were then calculated and collected 5 seconds before and 10 seconds prior to air puff administration. The mean of two traces were averaged together for each subject and then combined using custom MATLAB script (available upon request) to group individual data into groups. Group 465 nm signal data were then converted into 500ms bins for analysis to quantify differences between different conditions. All statistical analyses were conducted using GraphPad Prism 7. Five subjects were removed from the study due to lack of signal/cannula removal. Repeated measures one-way ANOVA analysis of variance (ANOVA) were used to determine differences between conditions for all groups in the air puff condition. Two-way ANOVA analyses were used to compare area under the curve between groups and time points. Paired t-tests were used to determine differences within groups between different time points in the binned trace and auditory-only analyses. Fisher's exact tests were used to compare percentages in animal darting behavior. Mixed effects two-way ANOVA analyses were used to compare time spent walking and rearing.

Competing Interests

The authors have no competing interests to report.

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