Title

Chronic Corticosterone Pretreatment Reverses Psilocybin's Effects on Mouse Anxious and Hedonic Behaviors

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Abstract

Psilocybin has shown positive preliminary signals in small-scale clinical trials for psychiatric disorders that exhibit maladaptive stress responses as a major component of their presentation. However, there are relatively few assessments of whether an acute administration of psilocybin exhibits reproducible effects in rodent models useful for the study of stress-associated psychiatric disorders. Here, we measured the responses of male C57BL/6J mice to this compound in a battery of relevant behavioral tests. These tests included the open-field test, forced swim test, sucrose preference test, and novelty suppressed feeding test. Furthermore, these tests were presented in either the absence or presence of chronic corticosterone administration, as a chemically induced model of ongoing stress burden. Our results indicate that the effects of psilocybin within these tests are dependent on the chronic hormonal stress burden of the mice: psilocybin alone promotes anxiolytic and hedonic responses, but promotes anxiogenic and anhedonic responses when pre-treated with chronic corticosterone. This identified interaction between stress hormone burden and psilocybin behavioral effects in mice suggests the possibility of further developing rodent behavioral models that can assess additional context-dependent effects of psychedelic administration that are deemed clinically-relevant, but are otherwise difficult to control for, in human studies.

Introduction

Investigations applying the serotonin 2A receptor (5-HT2A) agonist psilocybin as a psychiatric medication, have recently proliferated.[1–4] This classical psychedelic compound has been investigated in multiple clinical trials for substance use disorders (SUD), end-of-life anxiety, and major depressive disorder (MDD).[5–9] In the human studies carried out to date, psilocybin has reliably demonstrated improvements in primary outcome measures, with effects that occur rapidly and persist for a period of weeks to months. However, there remain several important factors to consider in regard to the rigorous interpretation of these positive preliminary results.

In accordance with ethical best-practice guidelines for this field, psilocybin is administered as one part of a supportive treatment regimen that includes intensive psychological preparation, debriefing sessions, as well as guided support and environmental controls, such as music exposure, during the administration session.[10,11] These non-pharmacologic interventions are presented in accordance with the 'set and setting' model, which is supported by repeated observations that both internally and externally generated stimuli are modifiers of the subjective effects of classical psychedelic compounds.[12,13] Therefore, studies of therapeutic interventions with psychedelics are intrinsically measuring the overall effects of this complete intervention package, rather than measuring the effectiveness of the compound itself in isolation. Furthermore, rapid unblinding due to obvious psychological effects makes placebo control difficult. Well-designed studies with psilocybin have variously attempted to mitigate this challenge, but large effect sizes are still observed in many of the 'inactive' conditions.[7,8] While these residual effects could represent efficacy of the non-pharmacologic interventions alone, they could also reasonably arise from subjects' therapeutic expectation biases and the associated 'meaning effect' arising from drug administration while enrolled in a psychedelic drug trial.[14,15].

For psychedelic therapeutic investigation, these issues are far from being mere technical caveats; intensity measurements taken across multiple phenomenological dimensions of altered states of consciousness have repeatedly demonstrated that specific types of experiences (variously characterized as 'peak', 'mystical', or 'ego-dissolving') are correlated with larger effect sizes in primary measures of clinical response.[3,6,15–20] However, classical psychedelics have also been demonstrated to induce both functional and structural synaptic plasticity in neuronal preparations from mammalian and non-mammalian sources.[21–27] While cellular and molecular observation of this plasticity in living human brain tissue is not currently feasible, these *in vitro* and *ex vivo* findings are consistent with the reductions in connectivity strength amongst established networks and increases in connectivity and signal entropy seen in human functional imaging studies. [1,28–30] Theoretical frameworks for the therapeutic actions of psychedelics in MDD have likened these molecular effects to a 'reset' that permits relaxation and de-weighting of prior beliefs along with reductions in behavioral and cognitive rigidity.[31] Critically, this framework does not necessarily imply that therapeutic benefits should occur in the absence of the active integration, informational processing, and narrative restructuring that form essential features of the clinical interventions used with psychedelics.

Intriguingly, animal models traditionally applied in the assessment of therapeutics for substance use disorders (SUD), major depressive disorder (MDD), and anxiety disorders seem to provide one means to help isolate drug effects from these psychological support effects. Nevertheless, studies designed to assess whether rodent behaviors will be rapidly and persistently sensitive to treatment with psilocybin and other 5-HT2A agonists have only

emerged relatively recently. [27,32–38] The results of these studies have been variable, and even somewhat conflicting, but the obvious differences amongst the drugs, dosing strategies, species, strains, and outcome measures used in this small sample make any systematic comparisons premature. More complex phenomena, such as drug-induced modification of learned associations across repeated tasks, have also been suggested as additional contributors to the variability amongst observed outcomes.[33] Therefore, identification of experimentally-modifiable biological and environmental factors that can influence rodent responses to psychedelics across functionally-relevant behavioral domains is warranted. As prior stress exposure is both a key feature of rodent models used to assess antidepressant-like activity and a proposed moderator of psilocybin's subjective effects in humans, this work tests the hypothesis that chronic pretreatment with the stress-associated hormone corticosterone will increase anxious responsiveness and decrease hedonic responsiveness in mice treated with psilocybin. [39–42]

Materials and Methods

Animals and Husbandry. All mice used (n = 270) in this work were acclimated to University of Wisconsin vivarium conditions for at least seven-days prior to handling or experimentation. Food pellets (LabDiet) and water (Inno-Vive) were available *ad libitum,* unless otherwise noted. All C57Bl6/J mice (male; 6-8 weeks old; The Jackson Laboratory, ME, USA) were housed in groups of three or four, while under a 12hr artificial light/dark cycle (light cycle from 6am-6pm; reversed for head twitch response and Experimental Battery 3). Room temperature remained constant between 22 – 24 °C. All experimental procedures were approved by the University of Wisconsin, Madison Animal Care and Use Committee (IACUC) and completed in full accordance with Research and Animal Resources and Compliance (RARC) guidelines.

Drugs. All controlled substances were handled by authorized users on the Schedule I, Schedule II-V DEA research licenses, and WI Special Use Authorizations held by Dr. Cody Wenthur. See Supplementary Materials and Methods for details on sources, preparation, and administration.

Behavioral Tasks. To measure acute (0 – 120 min post-injection) unconditioned responses to drugs, mice were assessed in an open field test (OFT) and using an automated HTR detection protocol adapted from previous approaches.[43,44] To measure post-acute (4 – 24 h post-injection) behavioral responses to drugs, mice were assessed in independent tests, including a forced swim test (FST), sucrose preference test (SPT), novelty suppressed feeding task (NSF) and OFT. For measurement of post-acute and delayed (2 – 7 days post-injection) drug effect sensitivity to oral exposure to 21-28 days of chronic corticosterone treatment, animals were tested in three independent Experimental Batteries incorporating the OFT, SPT, and NSF tasks. See Supplementary Materials and Methods for details of the conditions used for each of these tests.

Corticosterone ELISA. Following 28 days of chronic exposure to corticosterone or vehicle, and 15 min after the completion of the final OFT, animals were anesthetized with isoflurane and trunk blood was collected into microcentrifuge tubes following decapitation. The samples were then centrifuged at 10,000 RPM for 10 min, and stored at -80°C. Upon thawing, the plasma corticosterone concentrations were assessed using a colorimetric ELISA analysis (Enzo-Life Sciences, Corticosterone ELISA Kit) per the enclosed protocol. All samples were run in technical duplicate.

Statistical Analyses. Statistical analyses were performed using GraphPad Prism, version 7 (San Diego, CA). All tests were run as two-tailed analyses, setting $p < 0.05$ as the threshold for significance. Data analyzed across time were assessed using paired-analysis approaches; all samples were otherwise considered to be independent for analysis purposes. One and Two-way ANOVA analyses, as well as their non-parametric analogues, were corrected for multiple comparisons when assessing differences across each condition.

Results

In order to first identify a dose of psilocybin that could reliably demonstrate functional activity in 6-8 week old, male, C57Bl/6 mice, these animals were given IP injections with increasing dosages of psilocybin (0 – 3 mg/kg), and the acute effects of these compounds on non-conditioned behaviors were measured. Five minutes after injection their spontaneous locomotor activity was assessed in an OFT. Animals treated with 3 mg/kg of IP psilocybin demonstrated a significant increase in locomotor activity when compared to saline during the 10 min measurement period (Figure 1A; ANOVA with Sidak's correction for multiple comparisons: $p = 0.0042$) and a Dose x Time interaction was observed over the course of the observation (Figure 1B; Two-Way ANOVA: $F_{interaction}$ (60, 800) = 1.86; $p = 0.0001$).

To confirm that acute alterations in activity were occurring at this 3 mg/kg dose of psilocybin, animals treated under the same conditions were also observed for the induction of head-twitch responses (HTR), which are a classical proxy of hallucinogenic activity in mice. Head twitches were detected automatically using signals from magnets attached to existing implants (Figure 1C), using slight modifications from previously reported methods.[43,44] As validation that the 3 mg/kg dose was inducing acute disruptive effects in unconditioned responses *in vivo* during this immediate time period, the psilocybin-treated animals demonstrated a clear elevation in the number of head-twitch responses occurring in the 10 min after IP administration, as compared to saline or the dissociative-hypnotic ketamine (30 mg.kg) (Figure 1D; Kruskal Wallis with Tukey's: $p = 0.0006$). These head twitch responses rapidly returned to baseline during the full 2 h post-injection measurement period (Figure 1E; Two-Way ANOVA with Tukey's: $F_{interaction}$ (36, 360) = 3.967; $p < 0.0001$).

With this evidence of meaningful *in vivo* pharmacologic activity occurring at 3 mg/kg of IP psilocybin, the next step was to identify a post-acute time period where the acute locomotor and behavioral disruptions resulting from this dose had subsided, so as to minimize any interference with observations arising from behavioral tasks measuring anxious and hedonic responsiveness. To this end, animals treated with 0.3-3 mg/kg of IP psilocybin were again observed in the OFT for a ten-minute period, but this time, animals performed the test 4 h post-injection. At this post-acute 4 h time point, no differences were observed from saline for any of the doses tested (Figure S1A). Therefore, this 4 h delay was also used to assess whether there were disruptions in the post-acute behavioral responses of animals treated with 3 mg/kg of IP psilocybin in the Porsolt forced swim test (FST; Figure S1B-C)), a sucrose preference test (SPT; Figure S1D-E), and a novelty suppressed feeding test (NSF; Figure S1 F-G). No psilocybin-induced disruptions to coordinated motor, drinking, or feeding behaviors were apparent at this time point (Figure S1).

Having established an appropriate post-acute assessment window, the next step was a formal assessment of whether psilocybin's effects on anxious and hedonic responses were sensitive to chronic corticosterone exposure. The effects of 3 mg/kg IP psilocybin were measured in multiple experiments that measured OFT, SPT, and/or NSF behavioral alterations against a background of 28 days of chronic corticosterone exposure dosages at either 0 - 50

µg/mL delivered orally (PO), or 21 days of 0 - 80 µg/mL PO corticosterone (Figure 2). In all experimental designs, animals received only one dose of psilocybin, and the effects of this intervention were assessed at both post-acute (4h) and delayed (7 day) time points.

The effect of chronic corticosterone exposure on baseline behavioral changes was first measured prior to any introduction of additional drug challenge. It was found that the presentation of corticosterone in the animal's drinking water for 28 days resulted in a transient, non-significant reduction in animal weight gain (Figure3A-B), a significant reduction in the time spent in the center of an open field (Figure 3C; Student's t-test: $p = 0.009$) and a non-significant reduction in the distance traveled in that field (Figure 3D; $p = 0.055$), as well as a significant reduction in sucrose preference (Figure 3E; Student's t-test: $p = 0.002$). No change in total liquid consumption was observed following corticosterone treatment (Figure 3F). These results demonstrate that the chronic corticosterone administration paradigm used in these batteries was sufficient to increase anxious responsiveness and decrease hedonic responsiveness in the animals.

With confirmation of these effects from chronic corticosterone in hand, the behavioral effects of psilocybin were then measured at different levels of corticosterone exposure. These experiments used the relatively stressful NSF paradigm, which pits restriction-motivated feeding behavior against anxiety arising from a novel environment. In the NSF task, higher dosages of chronic corticosterone were found to significantly increase the latency to consume food (Two-Way ANOVA: $F_{Cort}(2, 54) = 12.55$; $p < 0.0001$), decrease the time in the central feeding zone (Two-Way ANOVA: $F_{Cort}(2, 54) = 32.23$; $p < 0.0001$), and decrease the total distance traveled in the NSF chamber (Two-Way ANOVA: $F_{Cort}(2, 54) = 21.04$; $p < 0.0001$) , which are observations consistent with the anxiogenic profile of corticosterone within the open field test (Figure 4A-C). At all tested levels of chronic corticosterone exposure, treatment with 3 mg/kg of psilocybin reduced the latency to first consumption of food (Figure 4A). Furthermore, when assessing the paired NSF feeding latency data between psilocybin and saline treated animals across all tested corticosterone conditions, these two conditions were found to have significantly different survival curves overall (Figure 4D; $p = 0.0435$). In contrast, psilocybin's effects on the time in the feeding zone were inconsistent across pretreatment conditions. In the absence of corticosterone exposure, psilocybin slightly increased time in the feeding zone, while at 80 µg/mL, it slightly decreased time in this zone (Figure 4B). This pattern does not appear to be driven by gross effects of psilocybin on movement in the NSF task (Figure 4C).

As a comparator compound that also demonstrates both rapid antidepressant activity and overt psychoactive effects in humans, but has a greater body of literature in measures of antidepressant-like activity in mice, as well as a divergent mechanism of action, 30 mg/kg IP ketamine was also administered. Administration for ketamine took place under the same chronic corticosterone ~~exposure~~ conditions as previously discussed for psilocybin above. Overall, ketamine displayed a similar pattern of activity when compared to psilocybin in the NSF assay (Figure 4E-H), including the non-significant interaction for time spent in the feeding zone at low versus high doses of corticosterone, but with a non-significant effect on the feeding survival curve observed at this 30 mg/kg dose.

In animals treated with either 0 or 50 µg/mL of corticosterone, the NSF task was further followed with an SPT assessment. This assessment took place the day immediately following psilocybin or ketamine treatment, along with assessments at set time intervals lasting for up to one-week post-injection. No effects on sucrose preference were observed for ketamine or psilocybin, regardless of previous corticosterone exposure (Figure S2A-D).

Considering that the stress from the NSF itself may have blunted the apparent magnitude of the interaction between psilocybin and chronic corticosterone, a final experiment was designed to assess both the post-acute and long-term interactions between these compounds, when administered in an otherwise comfortable environment. Animals were passively exposed to either vehicle or 80 µg/mL of chronic corticosterone PO for 21 days before being administered 3 mg/kg IP psilocybin. In this experiment, animals also had access to both sucrose and water in their home cage starting on day 14, and changes in sucrose preference were assessed using the SPT in the post-acute period following drug administration on day 21. Animals continued to have access to sucrose and water through day 28, and behavior in the OFT was then assessed at this time. Furthermore, in contrast to previous experiments, the animals in this cohort were tested during the dark phase of the light cycle, rather than the light phase, which provided a bigger signal window for measuring changes in distance traveled and time spent in the center zone of the OFT, regardless of psilocybin treatment condition (Figure S3A-E).

Using this paradigm, the sucrose preference of the animals was assessed in the post-acute period following psilocybin treatment. In corticosterone-exposed animals, an initially lower sucrose preference gradually increased above that of vehicle-exposed animals over the course of several days of exposure, and psilocybin was able to acutely reverse this effect (Figure 5A). No significant interaction was seen between psilocybin and corticosterone exposure in this task overall, due to a lack of observed effect for psilocybin on sucrose preference in the absence of this corticosterone pre-exposure (Figure 5B). No obvious longer-term effects of psilocybin were noted in regard to alteration of sucrose preference, when the animals' behavior was assessed daily for 7 days after treatment (Figure 5C).

On day 28, seven days after administration of 3 mg/kg IP psilocybin, these animals were next assessed in the OFT. At this time point, psilocybin-treated animals exposed to vehicle spent significantly more time in the center of the field than those previously exposed to chronic corticosterone (Figure 5D; ANOVA with Sidak's: $p = 0.007$). When this behavior is compared to the results from the saline-treated animals, a clear interaction between psilocybin and corticosterone effects is observed (Figure 5E; Two-Way ANOVA: $F_{interaction}$, $(1,28) = 5.95$; $p = 0.0213$). The observed trend for total distance traveled was similar, but non-significant (Figure 5F).

When the plasma from these animals was collected after completion of the open field test, the plasma corticosterone concentrations were interpolated onto a standard curve (Figure 5G), no direct interaction of psilocybin treatment on endogenous corticosterone concentrations was observed at this time (Figure 5H; Two-Way ANOVA: $F_{interaction}$ $(1, 28) = 0.80$; $p > 0.05$). Only the expected suppression of endogenous plasma corticosterone expressed due to feedback-inhibition was observed in those animals that had been chronically exposed to corticosterone PO (Figure 5I).

Discussion

These results demonstrate the presence of an interaction between chronic stress hormone burden and the behavioral effects arising due to administration of psilocybin in male C57BL/6J mice. When the stress-associated hormone corticosterone was delivered exogenously, psilocybin treated mice were found to exhibit anxiogenic behavioral patterns, while it was found to exhibit anxiolytic patterns in the absence of exogenous corticosterone supplementation. This interaction was most pronounced in the OFT results measured one week after psilocybin administration, but a similar pattern, albeit with smaller effect sizes, was

observed acutely in exploration of the NSF arena, as well as in hedonic responding to a 1% sucrose solution. Explicit assessment of sex differences in this response should be pursued in future work, particularly given prior observations regarding the differential actions of psilocin in male and female mice.[45] Interestingly, repeated corticosterone administration alone has recently demonstrated bidirectional effects on stress-associated behavioral adaptation; a single corticosterone exposure can enhance future anxiety-like responding, but a rapid stress or corticosterone re-exposure is sufficient to prevent this behavioral change.[46,47] As this complex pattern has been hypothesized to be dependent on delayed glutamate-sensitive neuroplastic effects occurring in the basolateral amygdala, future investigations into the mechanistic basis for the observed interaction between psilocybin and corticosterone will benefit from monitoring both immediate and long-term changes in corticosterone and glutamate concentrations following 5-HT2A agonist administration.[47,48]

The interaction of psilocybin with corticosterone treatment in mice observed here is also reminiscent of observations made in humans where the reported qualities of the drug effect are contingent upon the subject's comfort with the administration setting and their subjective expectations of the resulting hallucinatory experience.[40] Perhaps even more importantly, this observation also provides a potential conceptual and mechanistic link between the transiently increased plasma cortisol and transiently elevated medial prefrontal cortical glutamate concentrations that have been independently measured in humans following exposure to high doses of psilocybin, and independently associated with subjectively anxiogenic and negatively-rated experiences.[49] Although these mouse and human observational situations are clearly not directly comparable, they do identify translational experimental approaches to assess the biological phenomena that influence the 'set and setting' paradigm commonly applied to clinical psychedelic administration studies. As suggested for the pre-clinical approaches above, plasma cortisol concentrations and/or extracellular glutamate signals could be measured in humans prior to and following psilocybin administration, then correlated with discrete phenomenological effects (e.g. 5D-ASC) and/or therapeutic outcome measures (e.g. HAM-D). Conversely, the effects of non-pharmacologic manipulations within an animal's environment (such as social interaction or behavioral enrichment measures) during the time of psilocybin administration could also be assessed in regard to corticosterone concentrations, glutamate release, and subsequent behavioral responses. Given that such Environment x Drug effects have been reported in mice after the administration of selective serotonin reuptake inhibitors, this approach would make for a particularly useful comparison with approved antidepressant compound classes.[50]

In addition to the interaction between corticosterone and psilocybin highlighting the relevance of biological (in addition to psychological) processes on the outcome of psychedelic administration, these results also identify the NSF task as a previously-unreported mouse behavioral paradigm in which psilocybin exhibits an effect profile classically associated with antidepressant compounds. Indeed, psilocybin's pattern of overall activity in this task was similar to that of ketamine (albeit in a single-dose assessment), which is another rapidly-acting antidepressant with overt psychoactive effects. Notably, as opposed to the anxiety-like measures mentioned above, a reduction in latency to first feed in the NSF was observed across all tested chronic corticosterone exposure paradigms. Given that psilocybin's effects on hedonic and anxious responding appeared to otherwise be sensitive to corticosterone dose manipulations, direct measurements in other functional domains relevant to this feeding outcome, such as incentive motivation, are warranted. The results reported here further imply that future NSF observations monitoring exploratory and anxious measures may be more likely to yield robust results if the task is presented during the active (dark) phase of the mouse circadian cycle.

Looking beyond the NSF task, while modest, corticosterone-sensitive, acute effects on sucrose preference were observed following psilocybin administration, no long-term effects on SPT performance were observed here. Alternative mouse strains may be more appropriate for these measurements, as C57BL/6J mice demonstrate relatively low sensitivity to chronic corticosterone treatment and sucrose reward in this paradigm overall.[51] Furthermore, in agreement with previous studies in Flinders Sensitive Rats, we observed no post-acute effects on immobility time in the FST.[52] The previous studies that have reported psilocybin-mediated effects on FST in mice used more remote time-points for measurement, only beginning at least 7 days after administration, which could account for this difference in outcomes.[33]

Noting this growing battery of animal behavioral tasks in which psilocybin has demonstrated effects consistent with those of validated antidepressant compounds is especially relevant for two reasons. First, because these tasks appear to be differentially sensitive at distinct time points following psilocybin administration (i.e. SPT, acute; NSF, post-acute; FST; delayed), they may be independently useful for understanding psilocybin's influence on biological processes occurring at each of these time points (e.g. SPT, perceptual distortion; NSF; recovery/consolidation; FST, plastic/structural changes). Second, these antidepressant-like effects are observed in a mammalian species that is not susceptible to the psychedelic messaging encoded within human socialization processes or subsequent development of expectation biases, and has limited evidence demonstrating self-awareness or narrative meta-cognition. This suggests that such psychological mechanisms alone are not necessarily exclusively responsible for the therapeutic effects seen with psychedelic treatments.[53]

While clinical observations have previously identified phenomenological /psychological constructs as correlates of psychedelic therapeutic responsiveness, the interaction between psilocybin and prior chronic corticosterone administration in mice as reported here (across a variety of behavioral and environmental contexts) demonstrates that baseline stress hormone burden should also be considered as a behaviorally-relevant modifying factor in mammalian studies of context-dependent psychedelic activity. Repeated stress hormone concentration monitoring may thus be a future direction for human studies of psychedelic-assisted psychotherapy, to assess its value as a quantitative biomarker for 'set and setting' in regard to therapeutic responsiveness.

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Supplementary Information

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Figures

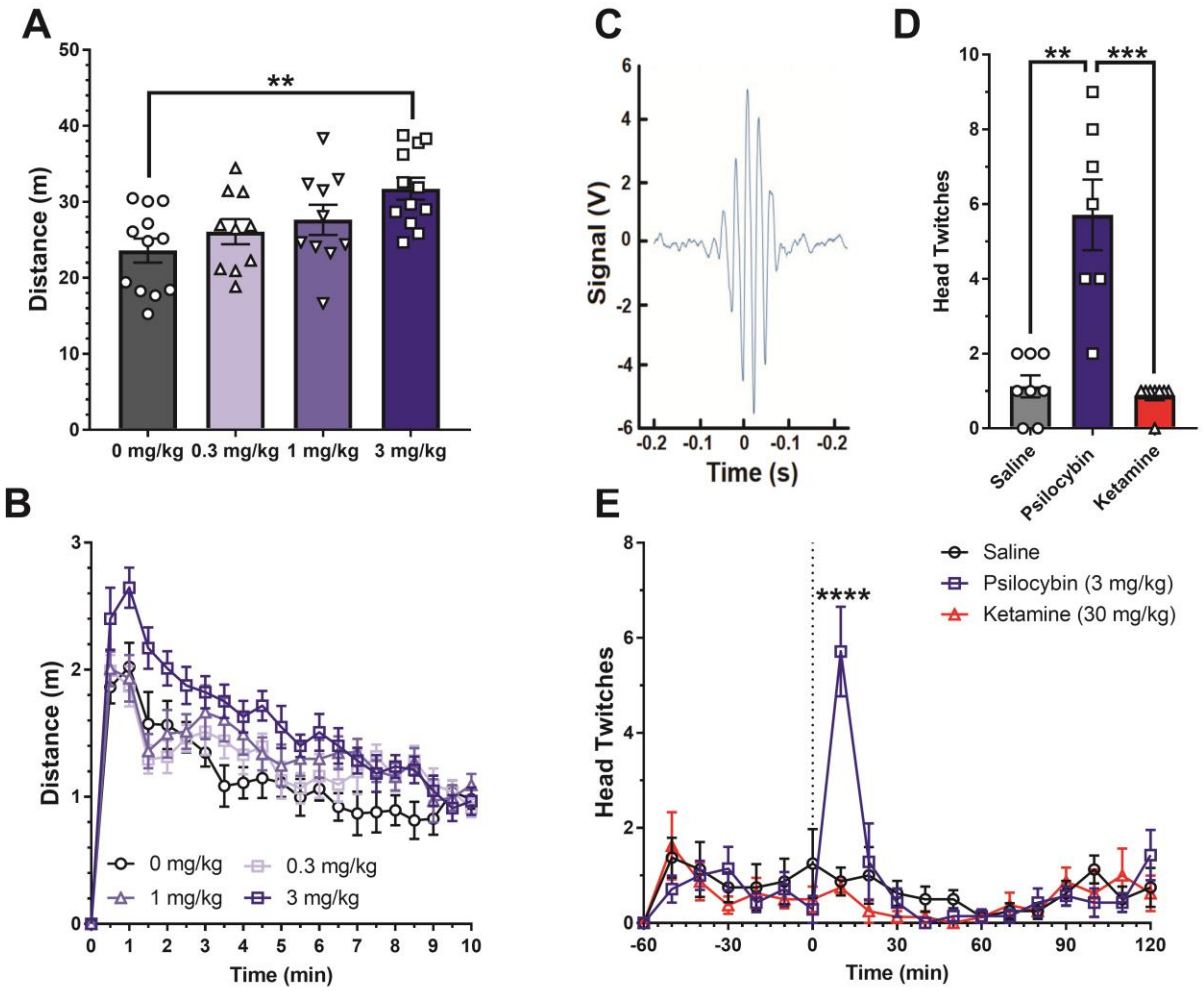

Figure 1. Intraperitoneal Psilocybin Acutely Modifies Locomotor and Head Twitch Behavior. A) Changes in locomotor response to increasing psilocybin doses in an open field test for 10 min post-injection. **: $p < 0.01$, ANOVA with Sidak's. B) Distance traveled every 30 s for the duration of the same open field test. C) Exemplar magnetometer signal for detection of head twitch response. D) Acutely increased head twitch responses for 10 min post-injection with 3 mg/kg psilocybin or 30 mg/kg ketamine. **: $p < 0.01$, ***: $p<0.001$, Kruskal-Wallis with Dunn's. E) Head twitches across each 10 min time period from 1 h before to 2 h after administration. (n=8, saline, ketamine; n=7, psilocybin) ****: $p < 0.0001$, Two-Way ANOVA with Tukey's. All error bars presented as mean ± SEM.

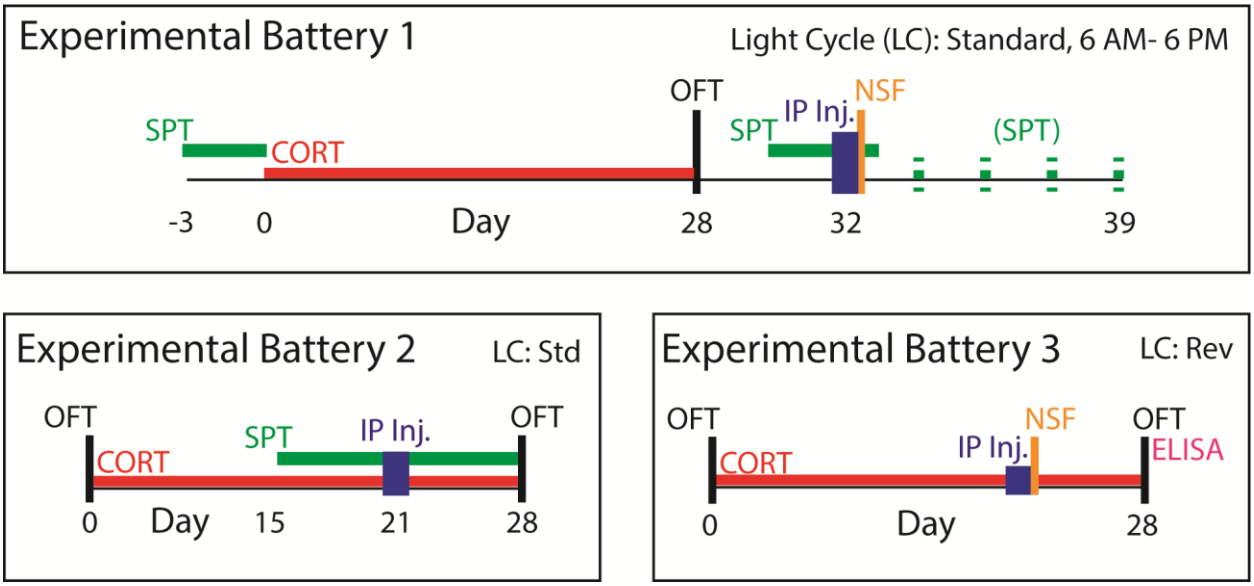

Figure 2. Timelines for Experimental Batteries Used to Assess Interactions of Psilocybin and Chronic Corticosterone. The first day of corticosterone (CORT) exposure is set as study day 0 for all experimental batteries. Definitions of events occurring in each timeline are as follows: SPT: Sucrose Preference Test; OFT: Open Field Test; IP Inj.: Intraperitoneal injection; NSF: Novelty Suppressed Feeding Task; ELISA: Enzyme-linked Immunosorbent Assay. In Experimental Battery 1, (SPT) and dashed bars denote that individual animals were retested at set time intervals post-injection in the SPT on either day 33, 35, 37, or 39. Standard light cycle (Std): lights on 6 AM – 6 PM. Reverse light cycle (Rev): lights on 6 PM – 6 AM.

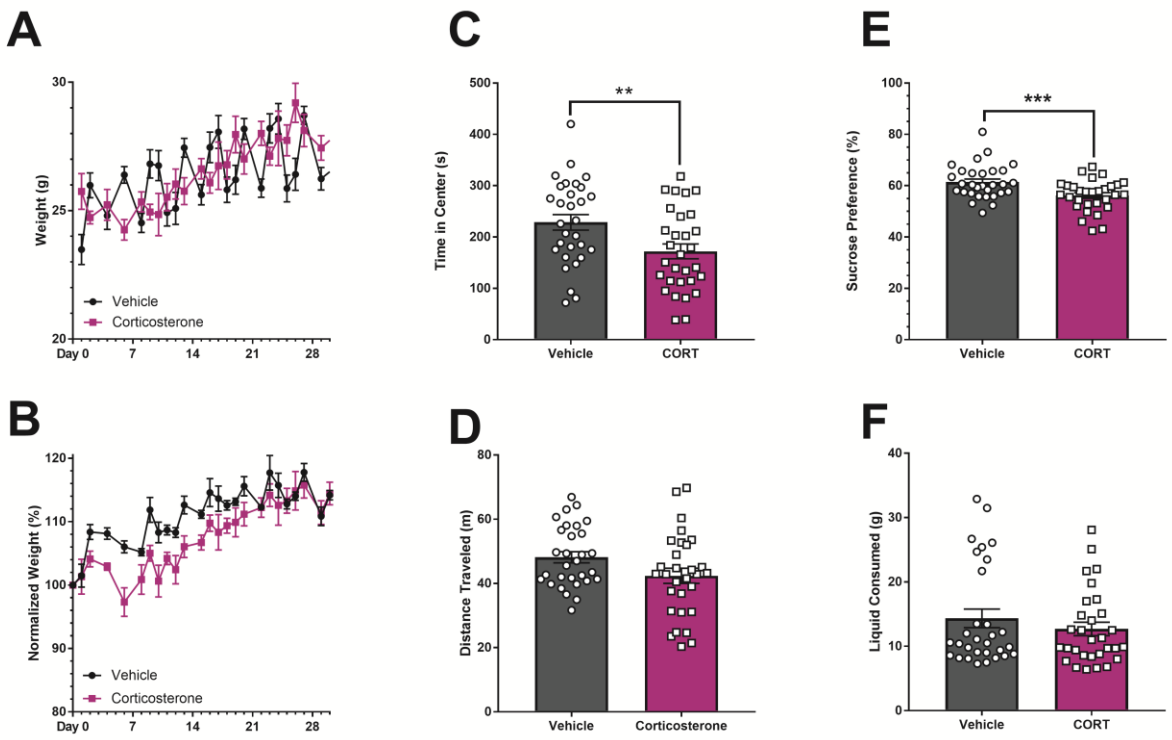

Figure 3. Chronic Corticosterone Exposure Via Drinking Water Yields Behavioral and Physiologic Changes in Mice. A) Weight measurements across the 28-day corticosterone exposure period (Vehicle, n = 30; Corticosterone, n = 30). B) Baseline-normalized changes in weight across the same period. C) Time spent in the center of the open field apparatus following 28-day corticosterone exposure period. **: $p<0.01$, T-test. D) Total distance traveled in the same open field test. E) Sucrose preference following a 28-day corticosterone exposure period. ***: $p<0.001$, T-test. F) Average of total liquid consumed on days 30 – 31 after chronic corticosterone exposure. All error bars presented as mean ± SEM.

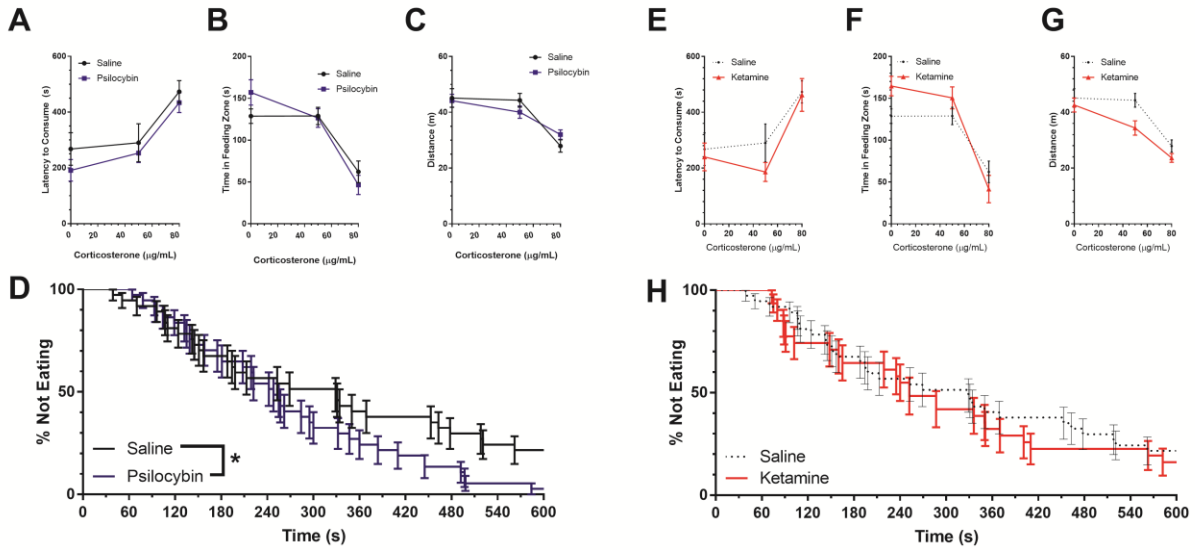

Figure 4. Psilocybin Decreases Latency to Feed in the Novelty Suppressed Feeding Task Across All Corticosterone Exposure Conditions. A) Latency to first chow consumption in the novelty suppressed feeding task following psilocybin administration and chronic corticosterone exposure (All conditions, n = 10). B) Time spent in the feeding zone in the same task. C) Total distance traveled in the same task. D) Survival curves of latency to feed for saline and psilocybin as matched across all corticosterone conditions (Saline, n = 36; Psilocybin, n = 36) *: $p < 0.05$, Log-Rank Test. E-H) Responses to 30 mg/kg intraperitoneal ketamine (n=30) in the same tasks and measurements as reported in A-D. Saline data replicated from A-D as dotted line for ease of comparison. All error bars presented as Mean ± SEM.

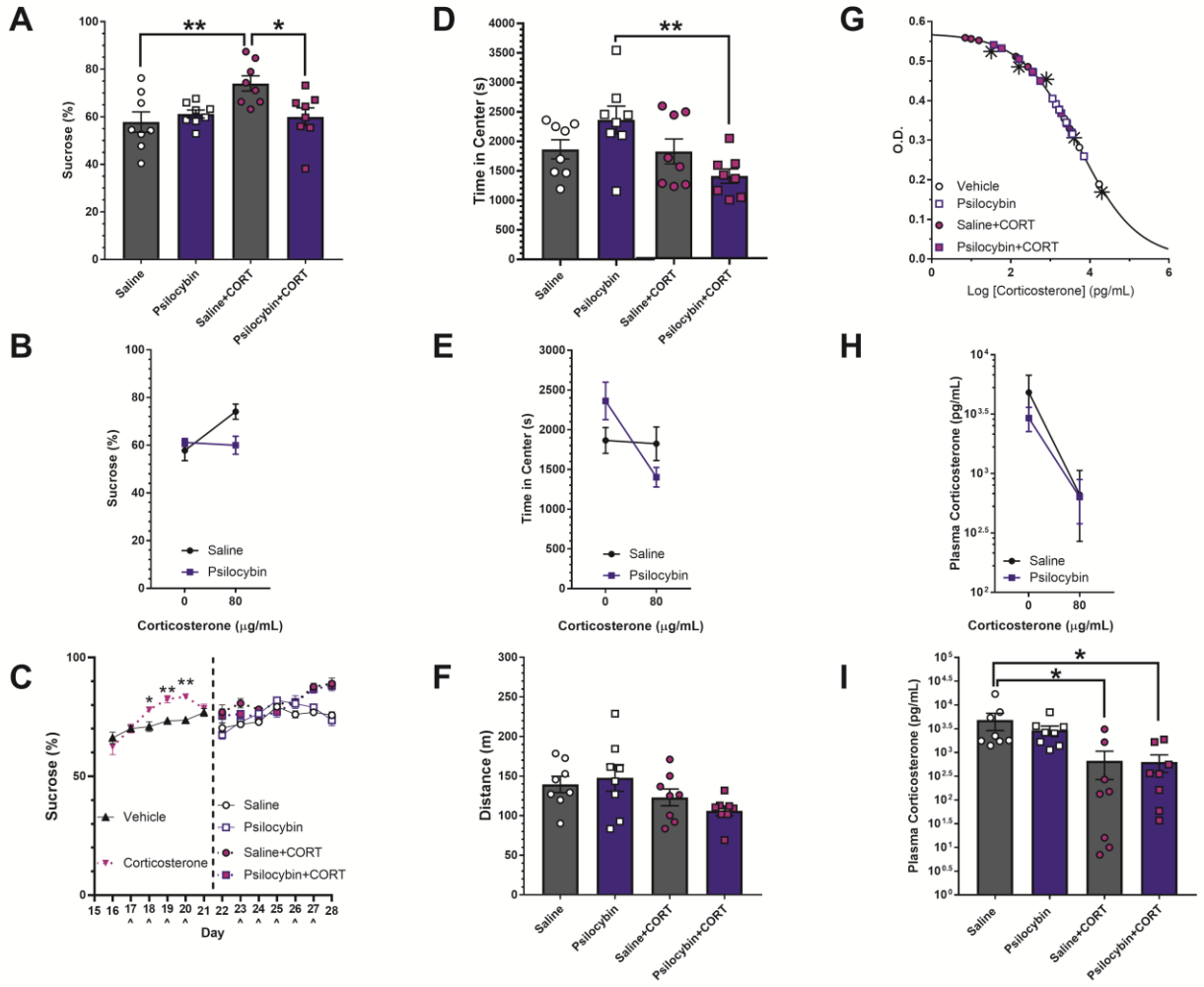

Figure 5. Psilocybin's Long-Term Anxiolytic-like Effects Become Anxiogenic When Combined with Chronic Corticosterone Exposure. A) Sucrose preference in vehicle or corticosterone-exposed animals during the period from 0 – 4 h following psilocybin administration. *: $p < 0.05$, **: $p < 0.01$, ANOVA with Sidak's. B) Interaction of psilocybin and corticosterone effects from the same test. C) Sucrose preference during the 6 days prior to psilocybin administration (Vehicle, n=16; Corticosterone, n = 15), and during the 7 days after psilocybin administration (Saline+CORT, n = 7, All other groups, n=8). ^: 3-day rolling average. D) Time in center of open field apparatus during a 150 min test period occurring 7 days following psilocybin administration. **, $P<0.01$, ANOVA with Sidak's. E) Interaction of psilocybin and corticosterone effects from the same test. F) Total distance traveled in the same open field test. G) Individual samples plotted onto standard curve of plasma corticosterone concentration as measured by competitive ELISA. H) Interaction of psilocybin and chronic oral corticosterone effects on plasma corticosterone measurements from these samples. I) Plasma corticosterone measurements by group from these samples. *: $p<0.05$, ANOVA with Sidak's.

SUPPLEMENTARY INFORMATION

Chronic Corticosterone Pretreatment Reverses Psilocybin's Effects on Mouse Anxious and Hedonic Behaviors

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Table of Contents

Supplementary Methods and Materials **2**

Surgery 2

Drug Preparation and Administration 2

Open Field Test (OFT) 2

Head Twitch Response (HTR) 3

Forced Swim Test (FST) 3

Sucrose Preference Test (SPT) 3

Novelty Suppressed Feeding (NSF) 4

Supplementary Figures **5**

Figure S1. 5

Figure S2. 6

Figure S3. 7

Supplementary Methods and Materials

Surgery

Skull screw EEG electrodes were chronically implanted in animals under isoflurane anesthesia (1.5 - 2%) using aseptic technique. Electrodes were placed bilaterally in the frontal (1.5 mm anterior to Bregma, 1.5 mm lateral to midline) and parietal (2.0 mm posterior to Bregma, 2.0 mm lateral to the midline) plates. Bilateral reference electrode screws were placed through the occipital plate and tied together to ground. EEG wires were stranded copper (0.012" diameter, 0.022" diameter including insulation; Cooner Wire, Chatsworth, CA). Wires were soldered to a 1-cm2 electrode interface board (EIB-16; Neuralynx, Bozeman, MT), which was fixed into place using dental cement (Fusio A3; Pentron; Orange, CA). Animals recovered for at least 5 days prior to the first recording day and were housed individually.

Drug Preparation and Administration

Psilocybin powder (USONA Institute; Madison, WI) was diluted in 0.9% sterile saline, then acidified to a pH of 1-2 with 1 M HCl, sonicated for 30-60s, and brought to pH 6-7 using 1 M NaOH. This material was filtered through a 0.2 um filter, and administered intraperitoneally (IP) at doses between 0.3 – 3 mg/kg. Ketamine Hydrochloride (Spectrum Chemical Mfg. Corp.; Gardena, CA) was diluted in 0.9% sterile saline, filtered through a 0.2 um filter, and administered at a dose of 30 mg/kg IP. All IP injections were given at a volume of 10 mL/kg. Corticosterone (Sigma-Aldrich) was diluted in 10% ethanol (EtOH) or 4.5% (2-hydroxypropyl)-Beta-Cyclodextrin (Biosynth-Carbosynth) in water, vortexed for 1 min, and then sonicated for 3 min at 22 °C, before being diluted to either 1% EtOH or 0.45% Beta-cyclodextrin in the animals' drinking water. For experimental batteries requiring chronic corticosterone exposure, mice were given ad libitum access to either corticosterone water (Battery 1, 50 µg/mL; Batteries 2 and 3, 80 µg/mL) or vehicle (1% EtOH, Battery 1; 0.45% (2-hydroxypropyl)-Beta-Cyclodextrin, Batteries 2 and 3) in their home cage for 28 days. [44–46]. Both vehicle and corticosterone bottles were refreshed every 7 days for the duration of the 28-day period.

Open Field Test (OFT)

To test for drug-induced changes in locomotor, exploratory, and anxious behavior, mice were assessed in the OFT. Mice were injected IP with psilocybin (0 - 3 mg/kg) and then individually placed into a corner within an open-field apparatus (41x20x24 cm), at a time period from 5 min (Acute, Baseline), 4 h (Post-Acute, Batteries 1-3) and/or 7 days afterward (Batteries 2 and 3). The center zone was defined as the middle one-third (6x27cm) of the arena. The apparatus was illuminated at 250 lux. In acute and post-acute measurements, mice were allowed to explore freely for 10 minutes, and in Battery 1, mice were allowed to explore freely for 30 min. For Batteries 2 and 3, mice were allowed to habituate for 60-minutes before being administered an IP injection. The mice were then placed back into their respective open-field arena for an additional 90-min period. Time spent in the center and total distance travelled were automatically quantified using the Any-Maze software. Each apparatus was cleaned before and after each test with Trifectant. All OFT measurements were run between 1-4 PM, which was the light phase of the cycle for all experiments other than Battery 3.

Head Twitch Response (HTR)

To measure acute unconditioned responses to psilocybin, mice were assessed using an automated HTR detection platform adapted from previous approaches.[47,48] In this study, mice already implanted with chronic skull screw EEG electrodes were anesthetized with isoflurane (1.5 - 2%) to attach a neodymium magnet to the exposed dental cement from the EEG implant, at least 1 day prior to recording. Following recovery, individual animals were placed into a clear acrylic cylinder (15.24 cm height x 15.24 cm diameter) wrapped with ~300 rotations of 30 Gauge copper magnet wire, (Essex, Fort Wayne, IN) inside of a dark sound-attenuation chamber, connected to a flexible tether (ZC16) to record EEG while allowing free range of motion, and their behavior was recorded using an infrared camera (240x320 pixels) controlled by Synapse (Tucker Davis Technologies, Alachua, FL [TDT]) for 1 h prior to drug administration. Magnetometer signals were amplified near the source with a homemade custom circuit, and the signal was routed to an RZ5D (filtered at 0.2-1000 Hz, then digitized at 3,051.8 Hz). After this time period, the animals were administered 3 mg/kg IP psilocybin and recorded for an additional 4 h. Changes in the local magnetic field induced by head twitches (~ 60-90 Hz signal) were assessed using in-house MATLAB code. Automated results were compared to observed HTRs for internal validation.

Forced Swim Test (FST)

To measure the post-acute effects of drug treatment on immediate threat response, mice were assessed in the FST. Mice were injected IP with either psilocybin (3 mg/kg), ketamine (30 mg/kg) or saline. Animals were individually placed into a clear Plexiglas swim tank (46x10cm) for a period of 6 min. Water temperature was maintained at 26°C and the tanks were illuminated at 40-42 lux. Immobility time and distance travelled were quantified for the final 4 min of the test using Any-Maze software. At the completion of the testing period, animals were removed from the water, dried and placed into a clean cage with a heating pad to facilitate rapid recovery of normal body temperature. The animals were monitored in this chamber for 15-minutes before being placed back into group housing. All FST measurements were run during the light phase of the light cycle, between 1-4 PM.

Sucrose Preference Test (SPT)

The SPT was conducted as a measure of hedonic responding both acutely and chronically. In this test, mice were placed into individual housing (41x20x24cm) to habituate for 48 h and then provided two identical bottles with either a 1% sucrose solution or water. At this time, mice had ad libitum access to standard chow and the 2 bottles provided. For post-acute baseline measurements and Experimental Battery 1, animals underwent three 16 h restriction periods during which they had access to the 1% sucrose solution and water, with food and water provided between these periods. Immediately after the final restriction period, two identical bottles containing either 1% sucrose solution or water were placed into each cage for an additional 16 h. For Experimental Battery 1, animals were returned to group housing. For Experimental Batteries 1 and 3, animals were treated with 0 – 80 µg/ml of chronic corticosterone for 28 days in group housing. For Experimental Battery 1, the SPT was repeated

on day 21 under the same baseline conditions as listed above. For Experimental Battery 3, animals were placed into individual housing and given continuous access to 1% sucrose, water, and food for 6 days (starting on day 15), followed by administration of psilocybin or saline, and another 7 days of continuous access to 1% sucrose, water, and food (through day 28). At this time both bottles were weighed daily. For animals being treated with chronic corticosterone during this continuous access period, both the 1% sucrose and water bottles retained the same corticosterone or vehicle concentrations as the rest of the 28-day exposure. The bottles were weighed at the end of each test period, and sucrose preference was calculated as: sucrose weight / (sucrose weight + water weight) *100.

Novelty Suppressed Feeding (NSF)

In order to measure animal behavior in a task combining motivated behavior with anxious responding, mice were assessed within the NSF. Animals underwent a sequential food reduction of 2-Days at 20% and 1-day at 80% (Post-acute), or food deprivation (24 h, Battery 1; 16 h, Battery 2). Mice then received an IP injection of saline or drug 4-5 h prior to performing the NSF test. For the test, a food pellet soaked in 50% sucrose solution was placed into a glass petri dish that served as the feeding zone (9x0.375 cm) and centered within a novel cage environment (61x41x37cm) that was illuminated at 600 lux (Battery 1) or 960 lux (Battery 2). Mice were then placed into a corner of the apparatus and allowed to explore for 10 minutes. Latency to first feed was recorded by a trained and a blinded observer, and movement and distance traveled were monitored via the Any-Maze software. Pellet weights were also obtained immediately before and after each test. After testing, the mice were returned to their housing and given normal food and water. The Each apparatus was cleaned before and after each test with Trifectant. All NSF measurements were run during the light phase of the light cycle, between 11-4 PM.

Supplementary Figures

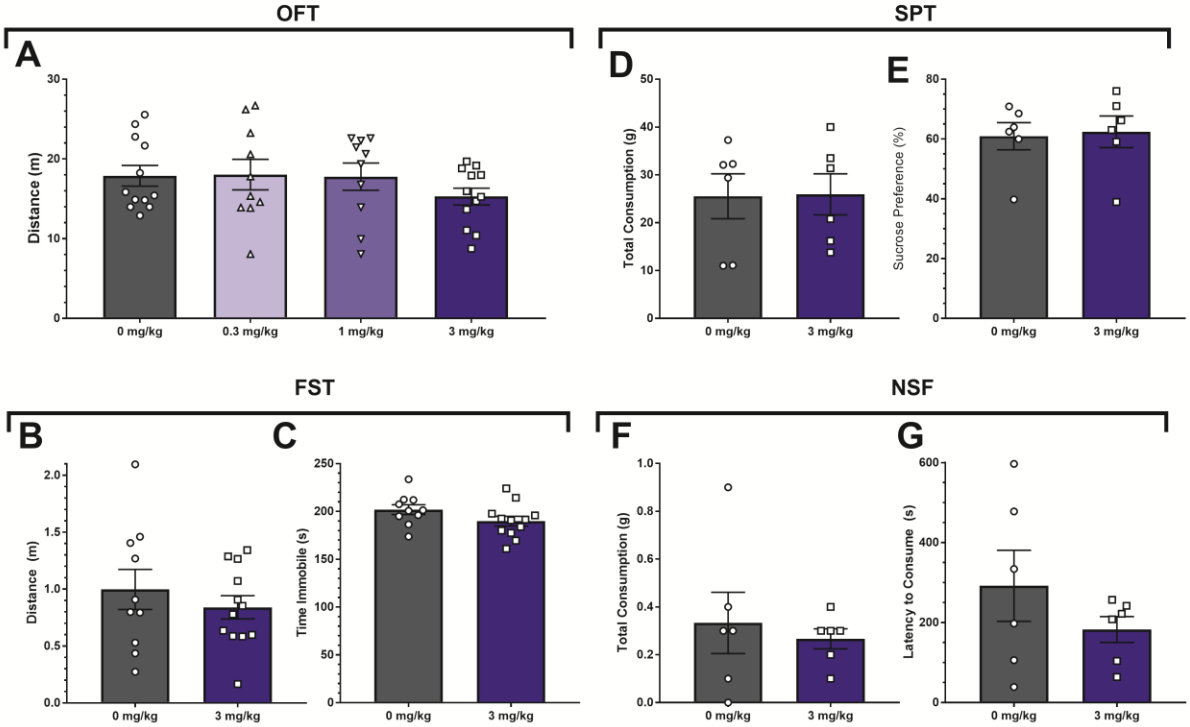

Figure S1. **No Motoric or Consumptive Disturbances of 3 mg/kg Intraperitoneal Psilocybin Are Observed Post-Acutely at 4 Hours After Administration.** A) Locomotor response to psilocybin doses in an open field test from 240-250 min after psilocybin injection. B) Total distance traveled in a forced swim test occurring 240 min after psilocybin injection. C) Immobility time from this forced swim test. D) Total liquid consumption (1% Sucrose and water) in a sucrose preference test for the period from 4 – 20 h after psilocybin injection. E) Sucrose preference from this sucrose preference test. F) Total chow consumption in a novelty suppressed feeding task for the period from 240-250 min after psilocybin injection. G) Latency to first consumption from this novelty suppressed feeding task. All error bars presented as mean ± SEM.

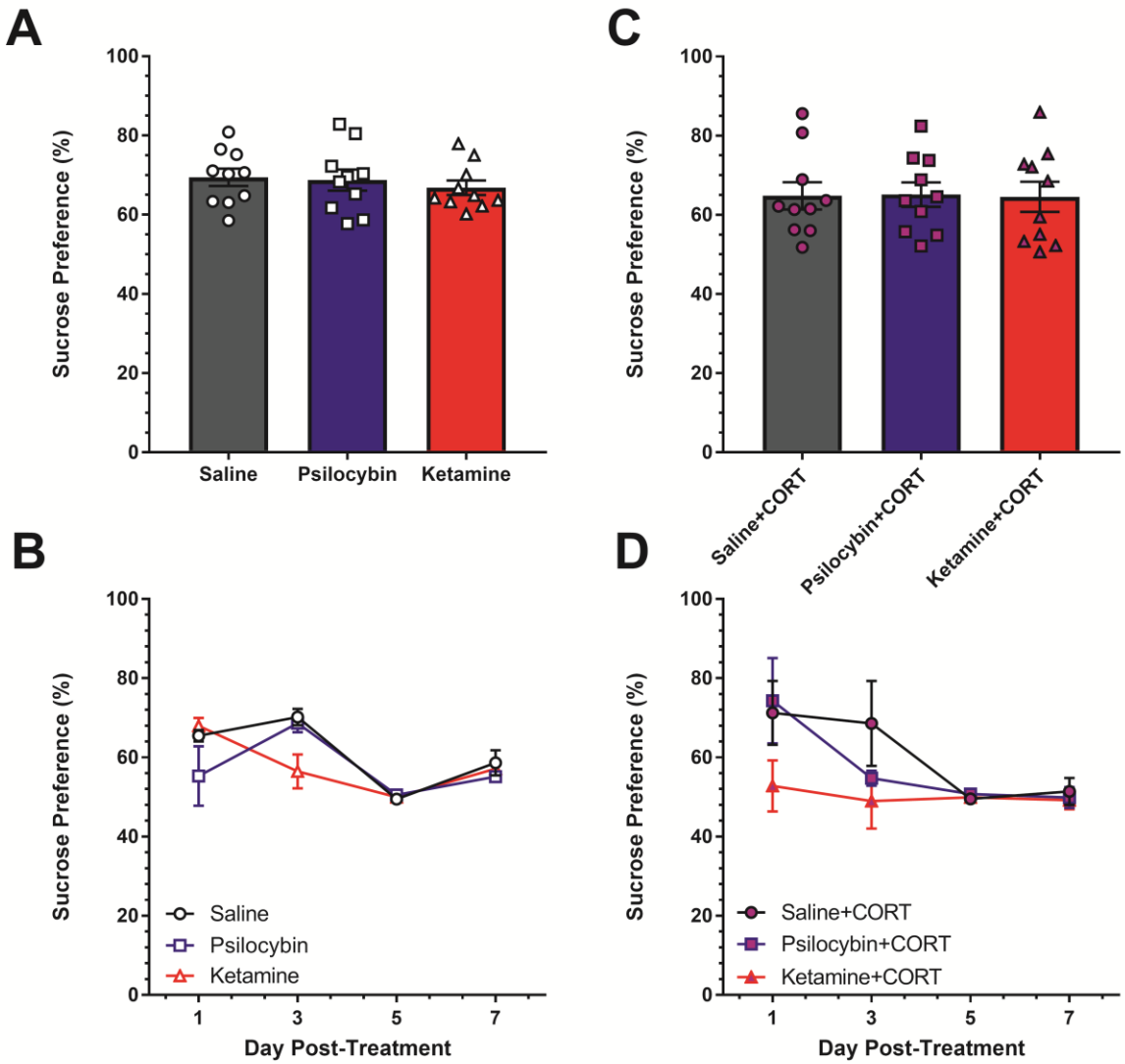

Figure S2. **No Long-Term Effects of Psilocybin and Ketamine on Sucrose Preference Behavior Are Observed in the Week Following Treatment.** A) Sucrose preference for 0-24 h period in drug-treated animals with chronic vehicle exposure. B) Sucrose preference of vehicle-treated animals upon retest beginning at 1, 3, 5, and 7 days after treatment (n= 2-3 per time point). C) Sucrose preference for 0-24 h period in drug-treated animals with chronic corticosterone exposure. D) Sucrose preference of corticosterone-treated animals upon retest beginning at 1, 3, 5, and 7 days after treatment (n= 2-3 per time point). All error bars presented as Mean ± SEM.

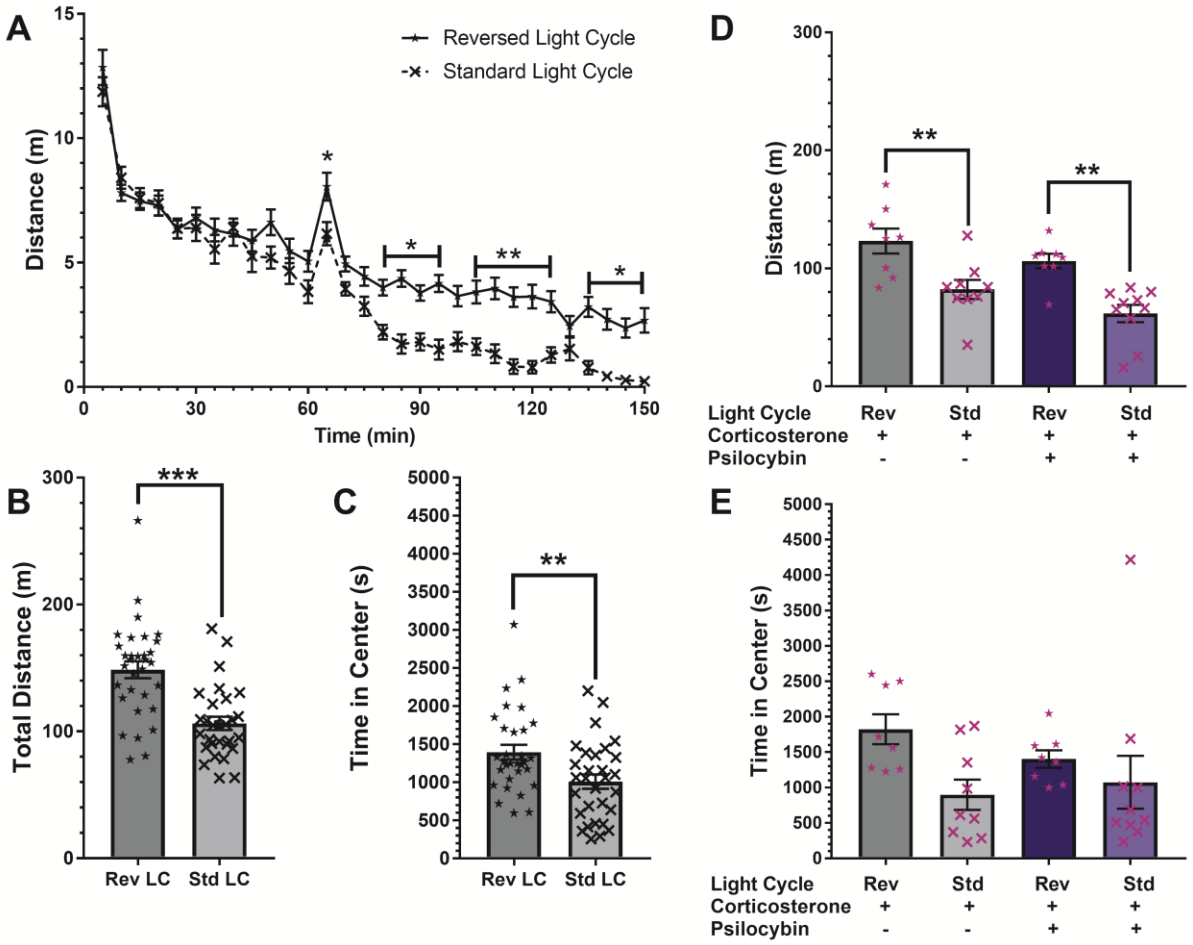

Figure S3. **Light Cycle Reversal Increases Signal Window for Locomotor and Center Time Measurements Across Treatment Conditions.** A) Distance traveled across 5 min time periods for 60 min prior to and 90 min after saline injection, across different light cycle conditions. (Standard, n = 30, Reversed, n = 32). *: p<0.05, ** p<0.01, Two-Way ANOVA with Sidak's. B) Total distance traveled in the same test. ***: p<0.001, T-test. C) Time in center of open field apparatus in the same test. **: p<0.01, T-test. D) Total distance traveled in an open field test from 5- 155 min after drug administration to corticosterone-exposed animals, across light cycle conditions. **: p<0.01, ANOVA with Sidak's. E) Time in center for the open field apparatus in the same test. All error bars presented as Mean ± SEM.