Enhancing cGMP signaling with psilocybin reduces head twitch and restructures the synaptic proteome while maintaining antidepressant response

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Abstract

New treatments for depression are needed that combine robust efficacy with improved scalability. Although psilocybin has demonstrated antidepressant effects in Phase 3 clinical trials, some of its psychedelic effects limit tolerability, necessitating administration in highly supervised clinical settings, and thus motivating development of serotonergic therapeutics that preserve antidepressant efficacy while reducing the acute psychedelic experience. We combined psilocybin with a phosphodiesterase-9 inhibitor (PDE9i), which raises cyclic GMP

levels, and observed substantial reduction in the mouse head twitch response (HTR) — a proxy for 5-HT_2A receptor-mediated psychedelic-like behavior in rodents — suggesting attenuation of acute psychedelic effects. Significantly, rescue of chronic stress-induced depressive-like behavior by psilocybin was maintained with the coadministration of PDE9i. Proteomic analysis of medial prefrontal cortex (mPFC) synaptosomes showed that the combination of PDE9i and psilocybin enhanced synaptogenesis pathways relative to psilocybin alone, while reducing pathways involved in G protein-coupled receptor (GPCR) signaling. Together, these results suggest that the combination of PDE9i and psilocybin may be a promising direction for psychedelic treatment, and point towards molecular pathways that dissociate acute psychedelic and antidepressant responses.

Introduction

Major Depressive Disorder affects over 300 million people worldwide and approximately one-third of them fail to respond to conventional antidepressants [1]. Psilocybin, a serotonin 2A receptor (5-HT_2AR) agonist, shows marked efficacy in refractory depression in phase 3 clinical trial (NCT05711940) [2]. Psilocybin administration is also accompanied by marked changes in perception mediated by 5-HT_2AR-dependent disruption of functional brain networks, including the default mode network [3]. The intensity of these acute psychedelic effects can limit broad clinical implementation, motivating efforts to dissociate therapeutic from acute psychedelic effects. Multiple strategies are being explored, including non-hallucinogenic analogs, biased agonists targeting specific 5-HT_2AR signaling pathways, and structure-based drug design approaches [4-6]. While these novel compounds show promise in preclinical studies, their clinical efficacy in patient populations remains unknown. Combination-based strategies offer an alternative approach that maintains psilocybin's therapeutic efficacy, while pharmacologically targeting mechanisms underlying its acute perceptual effects. Phosphodiesterase-9A (PDE9A) represents a compelling molecular target in this context. PDE9A is a high-affinity, cGMP-specific enzyme (Km ≈ 70 nM) with dense expression in hippocampus and prefrontal cortex, where it regulates cGMP pools

distinct from those controlled by other phosphodiesterases [7]. Selective PDE9 inhibitors, such as BAY 73-6691 and PF-04447943, enhance synaptic plasticity and produce robust pro-cognitive effects in rodent models [8]. Critically, PDE9 inhibition reverses mescaline-induced scratching behavior, a 5-HT_2AR-dependent response [9], providing direct evidence that cGMP modulation can influence behavioral outputs of 5-HT_2AR activation. Here, we investigated whether PDE9 inhibition modulates acute behavioral and molecular responses to psilocybin. We quantified head twitch response (HTR) as a behavioral readout of 5-HT_2AR engagement and predictor of acute perceptual effects, and assessed whether PDE9 inhibition interferes with psilocybin's antidepressant-like effects in a chronic stress paradigm. Finally, synaptic proteomic analysis was performed to characterize the molecular landscape underlying these combinatorial effects.

Methods

Animals and Housing

Male C57BL/6J mice (8–12 weeks old; The Jackson Laboratory) were group-housed under standard conditions (12:12 h light/dark cycle) with ad libitum food and water. All procedures were approved by the Yale University Institutional Animal Care and Use Committee.

Drugs

Psilocybin (item #14041, Cayman Chemical Company) was dissolved in saline and administered at 1 mg/kg, i.p. The PDE9A inhibitor PF-4181366 was synthesized and generously provided by Yuelian Xu at Chinglu Pharmaceutical Research LLC. It was analyzed by UPLC-MS at 98.95% purity. PF-4181366 was dissolved in 5% DMSO, 5% Tween-80 in saline at doses previously shown to increase cGMP levels in cortex and hippocampus (0.32, 1, 3.2, or 10 mg/kg, i.p.) [10].

The psilocybin dose selected here (1 mg/kg i.p.) is consistent with doses established to produce robust 5-HT_2AR-dependent HTR in rodents and to elicit neuroplastic responses in

preclinical depression models [11]; for reference, human therapeutic doses span approximately 0.14–0.36 mg/kg orally, a range shown to exhibit linear pharmacokinetics in healthy adults [12].

Head Twitch Response

We detected HTR using automated magnetometer-based system. Psilocybin (1 mg/kg) was administered 30 min after pretreatment with PF-4181366 (0.32, 1, 3.2, or 10 mg/kg) or vehicle and HTR was quantified immediately thereafter.

Locomotor Activity

Following 1h habituation, mice were placed individually into standard cages positioned within a 16-beam infrared photobeam grid (Med Associates Inc.). Locomotor activity was recorded for 60 min before and 60 min after injection of PF-4181366 (0.32, 1, 3.2, or 10 mg/kg) or saline.

Chronic Multimodal Stress

Chronic multimodal stress (CMMS) was adapted from Hesselgrave et al. [13]. Mice were single-housed and habituated to two-bottle choice (1.5% sucrose and water). Baseline sucrose preference was assessed on Days 2–3 using 1% sucrose during 14 h dark-phase sessions. Beginning Day 4, mice underwent 15 consecutive days of stress: 5 h daily restraint in ventilated 50 mL conical tubes under concurrent 2 Hz strobe lighting and 75 dB white noise. Session timing varied daily (8-11 AM). Sucrose preference was reassessed on Days 18–19 to evaluate stress-induced anhedonia.

Sucrose Preference Test

Sucrose preference was calculated as [sucrose intake / (sucrose + water intake)] × 100. A 1.5% solution was used during habituation; 1% was used for all testing sessions. Bottle positions were alternated to control for side preference.

Tail Suspension Test

On Day 17, mice were suspended by adhesive tape (1 cm from tail tip) to a horizontal metal bar. Behavior was video-recorded for 6 min, and immobility—defined as absence of active escape-oriented movements—was scored manually during minutes 1–5.

Pharmacological Treatment

Following TST, mice were stratified by sucrose preference and immobility into three groups: (1) vehicle–saline, (2) vehicle–psilocybin (1 mg/kg), and (3) PF-4181366 (10 mg/kg)–psilocybin (1 mg/kg). A 30 min interval separated pretreatment from psilocybin injection. PF-4181366 was administered intraperitoneally (i.p.) in 5% DMSO, 5% Tween-80, and saline; psilocybin was administered i.p. in sterile saline.

Post-Treatment Sucrose Preference

Four hours post-treatment, mice underwent a 14 h overnight sucrose preference test using 1% sucrose. Intake was measured by weight.

Forced Swim Test

On Day 21 (24 h post-treatment), mice were placed individually in cylindrical beakers filled with water (21 ± 1°C; 15 cm depth) for 6 min. Immobility—passive floating with minimal movements to maintain head above water—was scored during minutes 2–6.

Synaptosome isolation and LC-MS/MS

Mice were euthanized and the mPFC including ACC, PL and IL were dissected on ice. Crude synaptosomes were isolated as described in [14] and stored at -80 °C. LC-MS/MS data were acquired on a Thermo Scientific Orbitrap Fusion mass spectrometer coupled to a Waters M-Class UPLC system. Protein identification and label-free quantification (LFQ) were performed in Proteome Discoverer (Thermo Fisher Scientific, version 3.2.0.450) using standard processing and consensus workflows. Normalization between samples was based on total peptide amount and protein abundance ratios were calculated based on pairwise peptide ratios. Background-based t-tests were performed to identify significant differences in

protein abundances between conditions. Peptide- and protein-level FDR thresholds were set to 1% and only proteins with at least two unique peptides and those present in >50% of samples were considered for downstream analysis. Canonical pathway enrichment graphs and hierarchical clustering of pathways were generated through QIAGEN IPA (QIAGEN Inc., https://digitalinsights.qiagen.com/IPA).

Results

Pharmacological inhibition of PDE9 attenuates psilocybin-induced head-twitch responses without affecting locomotor activity.

Mice received psilocybin (1 mg/kg, i.p.) 30 min after vehicle or PDE9 inhibitor PF-4181366 pretreatment (0.32, 1, 3.2, or 10 mg/kg), and HTR was recorded for 30 min. ANOVA revealed a significant main effect of pretreatment on psilocybin-induced HTR ($F(4,23) = 6.042$, $p = 0.0018$). PF-4181366 at 3.2 mg/kg and 10 mg/kg significantly reduced HTR relative to vehicle ($p = 0.0097$ and $p = 0.0025$, respectively), whereas lower doses produced nonsignificant trends (Tukey's multiple comparisons test, both $p = 0.0532$). Expressed as percentage of vehicle control, PF-4181366 reduced psilocybin-induced HTR by approximately 47% at 0.32 and 1 mg/kg, 59% at 3.2 mg/kg, and 69% at 10 mg/kg, demonstrating robust, dose-dependent attenuation. To exclude nonspecific motor suppression, locomotor activity was assessed following PF-4181366 administration. Analysis revealed no significant effect of pretreatment on distance traveled ($F(4,24) = 0.2975$, $p = 0.8766$), with no differences between vehicle and any PF-4181366 dose (all adjusted $p \geq 0.96$). Normalization to baseline yielded comparable results ($F(4,24) = 0.8831$, $p = 0.4888$). These findings indicate that PDE9 inhibition selectively attenuates psilocybin-induced head-twitch behavior without affecting general locomotor activity, supporting specific modulation of 5-HT_2AR-mediated behavioral effects.

PDE9 inhibition does not interfere with the antidepressant-like effects of psilocybin in a chronic stress model.

Mice subjected to CMMS showed decreased sucrose preference across groups. Antidepressant-like behavior was assessed 24 h post-treatment using the forced swim test (FST). ANOVA revealed a significant treatment effect on immobility ($F(2,57) = 9.855$, $p = 0.0002$). Psilocybin significantly reduced immobility relative to saline-treated stressed controls ($p = 0.0008$). Critically, co-administration of PDE9 inhibitor did not attenuate this effect—the combination group similarly exhibited reduced immobility compared with saline controls ($p = 0.0004$).

Sucrose preference was assessed at baseline, post-CMMS, and post-treatment. Repeated-measures ANOVA revealed a significant effect of time ($p < 0.0001$), reflecting stress-induced reduction, but no treatment effect ($p = 0.5987$) or interaction ($p = 0.7661$).

These results indicate that psilocybin significantly reduces antidepressant-like behavior following chronic stress, and PDE9 inhibitor co-administration does not interfere with this effect. Sucrose preference was not significantly modulated by treatment at the examined time point.

Synaptosomal proteomics reveal that psilocybin/PDE9 inhibitor combination enhances synaptogenesis-related pathway compared to psilocybin alone.

Proteomic analysis of mPFC synaptosomal fractions collected 24h post-treatment revealed that PDE9i–psilocybin significantly altered synaptic protein composition relative to psilocybin alone. Pathway analysis showed an overall pattern of pathway downregulation with the PDE9i–psilocybin combination, including GPCR and serotonin receptor signaling. Remarkably, synaptogenesis, a process that is believed to be crucial for the therapeutic effects of psychedelics, was upregulated in the combination group. Pathways that were similarly regulated by PDE9i-psilocybin and psilocybin alone included CREB signaling, S100 family signaling and the NF1-Ras pathway. These pathways may provide mechanistic insight into the downstream pathways involved in psilocybin's therapeutic efficacy.

Conclusions

The present findings demonstrate that pharmacological inhibition of PDE9 selectively attenuates the acute head-twitch response to psilocybin without compromising its antidepressant-like effects in a chronic stress model. This behavioral dissociation is mechanistically supported by synaptosomal proteomic analysis showing preserved synaptogenic signaling pathways in the combination group, consistent with maintained therapeutic potential. The ability to reduce acute 5-HT_2A receptor-mediated behavioral responses while preserving antidepressant efficacy suggests that PDE9 inhibition may represent a viable strategy for improving the clinical tolerability of psilocybin. Notably, the 24-hour post-treatment timepoint selected for synaptosomal proteomics aligns with the established onset of psilocybin-induced neuroplastic consolidation in humans, where neuroimaging studies report changes in brain network connectivity and clinical symptom relief emerging within 24 hours of dosing and persisting for at least one month—providing translational grounding for the molecular window captured here [15]. It is possible that the increase in cGMP following PDE9 inhibition functions to block psilocybin-induced hyperexcitability and thereby reduce head-twitch responses. Collectively, these results support the broader hypothesis that combination-based approaches targeting intracellular signaling cascades can modulate the acute subjective profile of psychedelics, improve their tolerability while preserving their therapeutic mechanisms, thus making them scalable.

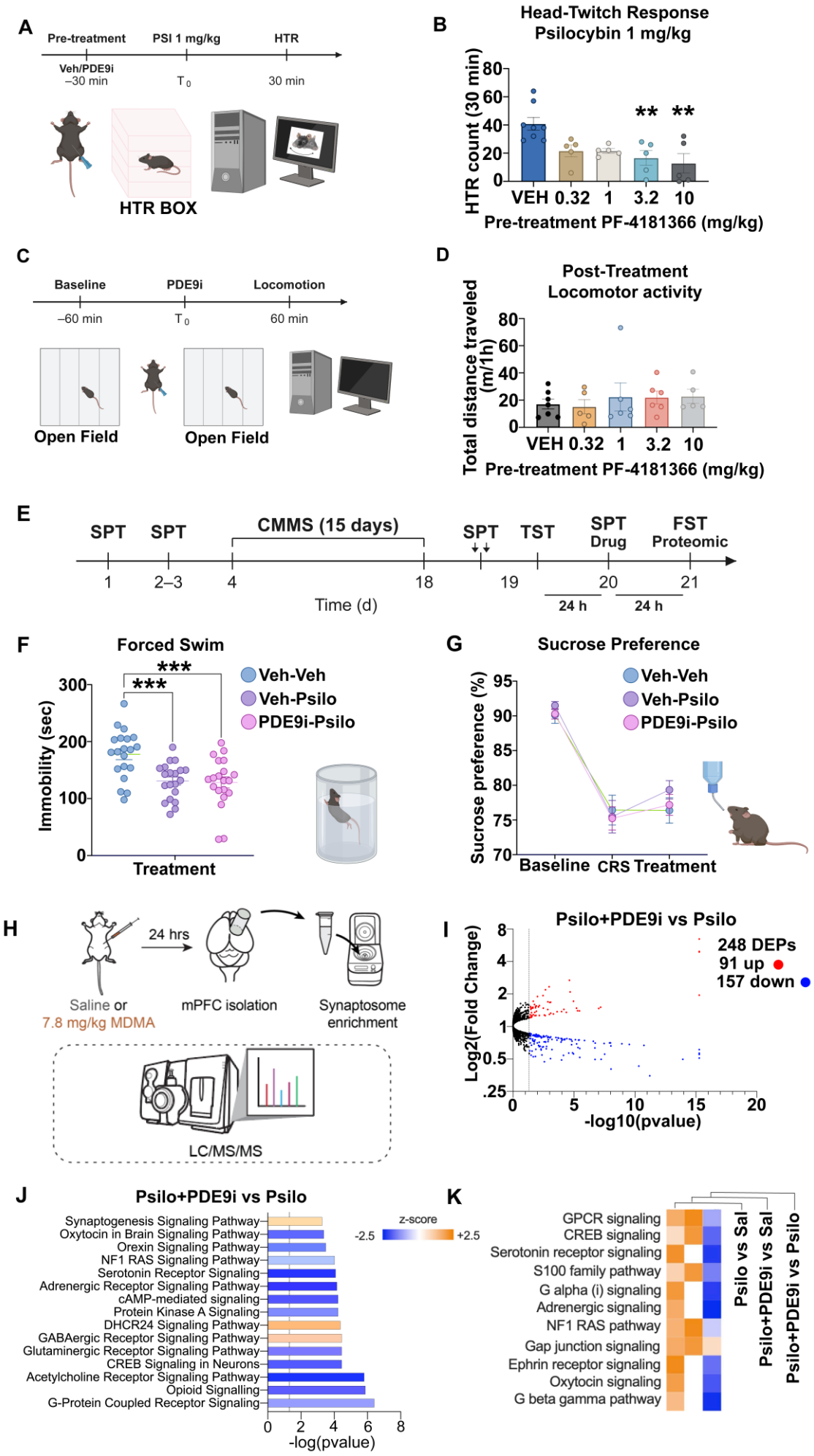
A
Pre-treatment
PSI 1 mg/kg
HTR
Veh/PDE9i
–30 min
T 0
30 min
HTR BOX
B
Head-Twitch Response
Psilocybin 1 mg/kg
HTR count (30 min)
80
60
40
20
0
** **
VEH 0.32 1 3.2 10
Pre-treatment PF-4181366 (mg/kg)
C
Baseline
PDE9i
Locomotion
–60 min
T 0
60 min
Open Field
Open Field
D
Post-Treatment
Locomotor activity
Total distance traveled (m/1h)
80
60
40
20
0
VEH 0.32 1 3.2 10
Pre-treatment PF-4181366 (mg/kg)
E
SPT
SPT
CMMS (15 days)
SPT
TST
SPT
Drug
FST
Proteomic
1
2–3
4
18
19
20
21
Time (d)
24 h
24 h
F
Forced Swim
***
***
Immobility (sec)
300
200
100
0
Treatment
Veh-Veh
Veh-Psilo
PDE9i-Psilo
G
Sucrose Preference
Sucrose preference (%)
95
90
85
80
75
70
Baseline
CRS Treatment
Veh-Veh
Veh-Psilo
PDE9i-Psilo
H
24 hrs
Saline or
7.8 mg/kg MDMA
mPFC isolation
Synaptosome
enrichment
LC/MS/MS
I
Psilo+PDE9i vs Psilo
248 DEPs
91 up
157 down
Log2(Fold Change)
8
4
2
1
0.5
.25
0
5
10
15
20
-log10(pvalue)
J
Psilo+PDE9i vs Psilo
Synaptogenesis Signaling Pathway
Oxytocin in Brain Signaling Pathway
Orexin Signaling Pathway
NF1 RAS Signaling Pathway
Serotonin Receptor Signaling
Adrenergic Receptor Signaling Pathway
cAMP-mediated signaling
Protein Kinase A Signaling
DHCR24 Signaling Pathway
GABAergic Receptor Signaling Pathway
Glutaminergic Receptor Signaling Pathway
CREB Signaling in Neurons
Acetylcholine Receptor Signaling Pathway
Opioid Signalling
G-Protein Coupled Receptor Signaling
z-score
-2.5
+2.5
0
2
4
6
8
-log(pvalue)
K
GPCR signaling
CREB signaling
Serotonin receptor signaling
S100 family pathway
G alpha (i) signaling
Adrenergic signaling
NF1 RAS pathway
Gap junction signaling
Ephrin receptor signaling
Oxytocin signaling
G beta gamma pathway
Psilo vs Sal
Psilo+PDE9i vs Sal
Psilo+PDE9i vs Psilo

Figure 1. PDE9 inhibition attenuates psilocybin-induced head-twitch responses without affecting locomotor activity or antidepressant efficacy, and preserves synaptogenic pathway engagement.

(A) Experimental timeline for head-twitch response (HTR) assessment. Mice received PF-4181366 or vehicle 30 minutes prior to psilocybin (1 mg/kg, i.p.) and HTR was recorded for 30 minutes in a dedicated HTR box. (B) Total HTR counts over 30 minutes showing dose-dependent attenuation following PF-4181366 pretreatment. Significant reductions were observed at 3.2 and 10 mg/kg compared to vehicle (**$p<0.01$; n=5–8/group). (C) Experimental timeline for locomotor activity assessment. Mice were placed in an open field 60 minutes before PF-4181366 treatment (baseline) and re-assessed for 60 minutes post-treatment. (D) Total distance traveled post-treatment showing no effect of PF-4181366 at any dose tested (n=5–7/group). (E) Experimental timeline for chronic multimodal stress (CMMS) and behavioral testing. Mice underwent SPT training (day 1), baseline SPT (days 2–3), 15 days of CMMS (days 4–18) with SPT on days 17–18, followed by TST (day 19), drug treatment with SPT (day 20), and FST (day 21; n=20/group). A separate cohort was used for synaptosomal proteomic analysis (n=6/group). (F) FST immobility time. Both psilocybin alone (Veh-Psilo) and in combination with PDE9 inhibitor (PDE9i-Psilo) significantly reduced immobility compared to vehicle-treated stressed controls (*** $p<0.001$). (G) Sucrose preference across baseline, post-stress (CRS), and post-treatment timepoints. (H) Schematic workflow for synaptosomal proteomic analysis. mPFC tissue was collected 24 h post-treatment, synaptosomal fractions were isolated, and proteins were quantified by LC/MS/MS. (I) Volcano plot of differentially expressed proteins (DEPs) in the psilocybin-PF-4181366 (Psilo+PDE9i) group versus Psilo alone (248 DEPs total; 91 upregulated, red; 157 downregulated, blue; n=6/group). (J) Ingenuity Pathway Analysis (IPA) of the Psilo+PDE9i vs. Psilo comparison showing significantly regulated canonical pathways ranked by −log(p-value). Bar color reflects predicted activation (orange) or inhibition (blue) based on z-score (scale: −2.5 to +2.5). (K) Heatmap comparing z-score-based pathway regulation across three contrasts (Psilo vs. Sal, Psilo+PDE9i vs. Sal, Psilo+PDE9i vs. Psilo). Data are

presented as mean ± SEM. HTR and locomotor data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. Forced swim data were analyzed by one-way ANOVA with Dunnett's post hoc test. Sucrose preference was analyzed using two-way repeated-measures ANOVA.

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