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

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Psilocybin produces antidepressant effects, but perceptual effects limit tolerability. We hypothesized that enhancing cGMP signaling with a phosphodiesterase-9 inhibitor (PDE9i) would diminish a rodent behavioral readout of 5-HT_{2A}R activation, while preserving antidepressant-like effects. PDE9i attenuated psilocybin-induced head-twitch while maintaining the antidepressant-like response. PDE9i–psilocybin reduced 5-HT_{2A}R-related pathways while enhancing synaptogenesis signaling.

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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_{2A}R) agonist, shows marked efficacy in refractory depression in phase 3 clinical trials (NCT05711940) [2]. Psilocybin administration is accompanied by marked changes in perception mediated by 5-HT_{2A}R activation [3]. These acute psychedelic effects could limit broad clinical implementation, motivating efforts to dissociate therapeutic from psychedelic effects. Multiple strategies are being explored, including non-hallucinogenic analogs and biased agonists targeting specific 5-HT_{2A}R signaling pathways [4, 5]. Combination-based strategies offer an alternative approach that maintains psilocybin's therapeutic efficacy, while pharmacologically targeting putative mechanisms that may underlie its acute perceptual effects. Phosphodiesterase-9A (PDE9A) represents a compelling target in this context. PDE9A is a high-affinity, cyclic guanosine monophosphate (cGMP)-specific enzyme ($K_m \approx 70$ nM) that is highly expressed in the hippocampus and prefrontal cortex, where it regulates cGMP pools that are distinct from those controlled by other phosphodiesterases [6]. Selective PDE9 inhibitors, including BAY 73-6691 and PF-04447943, have been shown to enhance synaptic plasticity and improve cognitive performance in rodent models [7]. PDE9 inhibition reverses 5-HT_{2A}R-dependent mescaline-induced scratching behavior [8], providing direct evidence that cGMP modulation can prevent 5-HT_{2A}R-dependent behavioral outputs. Here, we investigated whether PDE9 inhibition modulates

behavioral and molecular responses to psilocybin. We quantified head twitch response (HTR) as a behavioral readout of 5-HT_{2A}R engagement associated with the acute perceptual effects of serotonergic psychedelics. We also examined whether PDE9 inhibition interfered 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

C57BL/6J mice (8–12 weeks old; The Jackson Laboratory) were group-housed under standard conditions with ad libitum food and water. All procedures were approved by the Yale University Institutional Animal Care and Use Committee. HTR experiments included both male and female C57BL/6J mice, whereas chronic multimodal stress (CMMS) behavioral and proteomic experiments were performed in male mice, consistent with the validated CMMS paradigm [9].

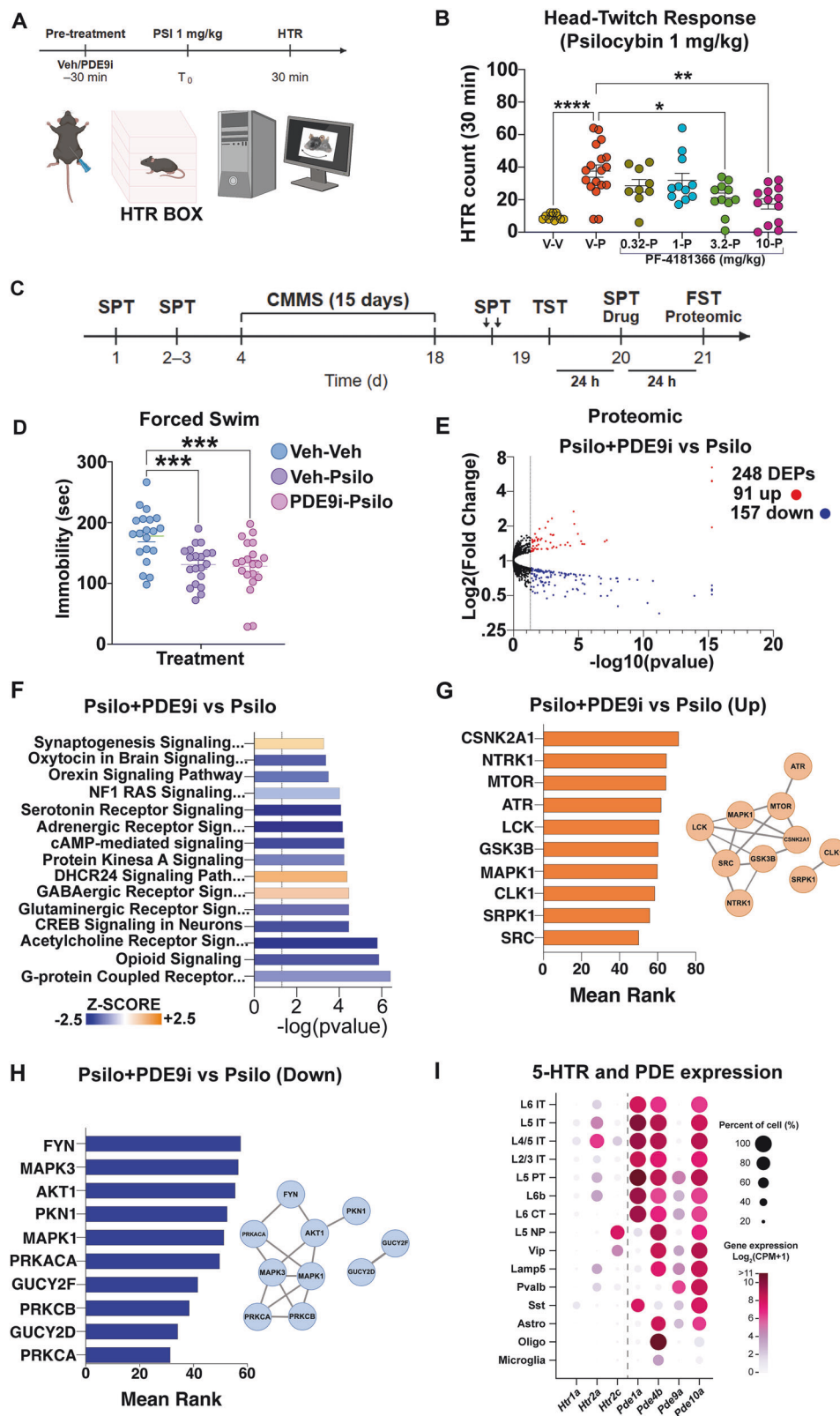
Drugs

Psilocybin (1 mg/kg, i.p.) or saline was administered 30 min after PF-4181366 (0.32–10 mg/kg, i.p.) or vehicle. All pretreatment injections used the same 5% DMSO/5% Tween-80 in saline formulation; animals not receiving PF-4181366 received matched vehicle. Drug preparation and dose-selection details are provided in the Supplementary Methods.

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Behavioral assays

The head-twitch response (HTR; $n = 9-18/\text{group}$) was quantified for 30 min using an automated magnetometer-based system [S3], and locomotor activity ($n = 5-7/\text{group}$) was recorded for 60 min. The CMMS cohort comprised $N = 78$ mice, divided into behavioral [$N = 60$ ($n = 20/\text{group}$)] and biochemical/proteomic [$N = 18$

($n = 6/\text{group}$)] cohorts; allocation and behavioral procedures are detailed in the Supplementary Methods.

Synaptosomal proteomics

Crude synaptosomes ($n = 6/\text{group}$) were isolated from pooled medial prefrontal cortex (mPFC), comprising the anterior cingulate

Fig. 1 PDE9 inhibition attenuates psilocybin-induced head-twitch responses without affecting antidepressant efficacy, and preserves synaptogenic signaling pathways. **A** Experimental timeline for head-twitch response (HTR) assessment. Mice received PF-4181366 or vehicle 30 min prior to psilocybin (1 mg/kg, i.p.) and HTR was recorded for 30 min in a dedicated HTR box. **B** Total HTR counts over 30 min in male and female mice showing dose-dependent attenuation following PF-4181366 pretreatment. Psilocybin significantly increased HTR relative to the vehicle–vehicle control, and significant reductions were observed at 3.2 and 10 mg/kg compared to vehicle–psilocybin ($*p < 0.05$, $**p < 0.01$, $***p < 0.0001$; $n = 9–18$ /group). Groups: V–V (vehicle–vehicle), V–P (vehicle–psilocybin), and PF-4181366 at 0.32, 1, 3.2, or 10 mg/kg followed by psilocybin (0.32-P, 1-P, 3.2-P, 10-P); psilocybin was administered at 1 mg/kg i.p. 30 min after pretreatment. **C** Experimental timeline for chronic multimodal stress (CMMS) and behavioral testing. Mice underwent sucrose-preference test (SPT) training (day 1), baseline SPT (days 2–3), 15 days of CMMS (days 4–18), post-stress SPT (days 18–19), tail-suspension test (TST; day 19), treatment with post-treatment SPT (day 20), and FST (day 21). The cohort sample size is $N = 78$: behavioral, $N = 60$ ($n = 20$ /group); biochemical/proteomic, $N = 18$ ($n = 6$ /group). **D** FST immobility time. Both psilocybin alone (Veh–Psilo) and in combination with PDE9i (PDE9i–Psilo) significantly reduced immobility compared to vehicle-treated stressed controls ($***p < 0.001$). **E** Volcano plot of differentially expressed synaptosomal proteins (DEPs) in the Psilo+PDE9i group versus Psilo alone (248 DEPs total; 91 upregulated, red; 157 downregulated, blue; $n = 6$ /group). **F** Ingenuity Pathway Analysis (IPA) of the Psilo+PDE9i vs. Psilo comparison showing significantly regulated canonical pathways ranked by $-\log(p\text{-value})$. Bar color reflects predicted activation (orange) or inhibition (blue) based on z-score (scale: -2.5 to $+2.5$). **G** Kinase Enrichment Analysis (KEA3) of upregulated DEPs in the Psilo+PDE9i vs. Psilo comparison. Inset: STRING network depicting known protein–protein interactions among predicted upstream kinases, including MTOR, MAPK1, ATR, LCK, SRC, GSK3B, CLK1, SRPK1, NTRK1, and CSNK2A1. **H** KEA3 analysis of downregulated DEPs in the Psilo+PDE9i vs. Psilo comparison. Inset: STRING network of interactions among predicted upstream kinases, including AKT1, FYN, PKN1, MAPK1, MAPK3, PRKCA, PRKCB, PRKACA, GUCY2F, and GUCY2D. **I** Dot plot showing expression of serotonin receptor genes (Htr2a, Htr2c) and phosphodiesterase isoforms (Pde4a, Pde4b, Pde9a, Pde10a) across major cortical cell classes in the mouse cortex (Allen Brain Cell Atlas). Dot size indicates the fraction of expressing cells, and color represents normalized gene expression ($\log_2(\text{CPM}+1)$). Data illustrate the enrichment of Pde9a and Htr2a in excitatory cortical neurons. Data are presented as mean $\pm$ SEM.

(ACC), prelimbic (PL), and infralimbic (IL) cortices 24 h post-treatment [S4] and analyzed by LC-MS/MS with label-free quantification. Differentially expressed proteins were subjected to pathway (IPA), kinase-enrichment (KEA3), and network (STRING) analyses; full instrument and analysis parameters are provided in Supplementary Methods.

Analysis of single-cell transcriptomics data

Allen Institute SmartSeq single-cell RNA-seq data were analyzed as described [10].

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 (PDE9i) PF-4181366 pretreatment (0.32, 1, 3.2, or 10 mg/kg), and HTR was recorded for 30 min. By Welch's ANOVA, psilocybin markedly increased HTR relative to vehicle–vehicle ($p < 0.0001$), and PF-4181366 dose-dependently attenuated psilocybin-induced HTR, reaching significance at 3.2 mg/kg ($p = 0.022$) and 10 mg/kg ($p = 0.003$), whereas lower doses did not differ from vehicle–psilocybin (0.32 mg/kg, $p = 0.57$; 1 mg/kg, $p = 0.92$). HTR values at 3.2 and 10 mg/kg were not significantly different compared to vehicle–vehicle group (Dunn's post hoc, adjusted $p = 0.66$ and $p = 1.00$, respectively). This attenuation was present in both sexes: a two-way ANOVA (pretreatment $\times$ sex) showed a significant main effect of pretreatment ($F(5,60) = 9.34$, $p < 0.0001$), no main effect of sex ($F(1,60) = 2.06$, $p = 0.16$), and no pretreatment $\times$ sex interaction ($F(5,60) = 1.57$, $p = 0.18$), consistent with comparable psilocybin-evoked head-twitch responses in male and female C57BL/6J mice [S5]. Locomotor activity was unaffected by PF-4181366 at any dose tested (one-way ANOVA, $F(4,24) = 0.30$, $p = 0.877$), indicating that HTR attenuation reflects specific modulation of 5-HT_{2A}R-mediated behavior rather than nonspecific motor suppression.

PDE9 inhibition does not interfere with the antidepressant-like effects of psilocybin in a chronic stress model. 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 controls ($p = 0.0008$). Critically, co-administration of PDE9i did not attenuate this effect—the combination group similarly exhibited reduced immobility

compared with saline controls ($p = 0.0004$). Thus, psilocybin significantly reduced FST immobility following chronic stress, and this effect was maintained with PDE9i co-administration. Longitudinal sucrose preference ($n = 26$ /group, $N = 78$) showed a significant effect of time ($F(1.629,122.2) = 83.53$, $p < 0.0001$), but no treatment effect ($F(2,75) = 0.55$, $p = 0.582$) or time $\times$ treatment interaction ($F(3.258,122.2) = 0.34$, $p = 0.813$). Tukey tests found no between-group differences at any time point (all adjusted $p \geq 0.3926$); sucrose preference was reduced after CMMS in all groups (all adjusted $p < 0.0001$) and was not altered by treatment. Cohort allocation is detailed in the Supplementary Methods.

Synaptosomal proteomics reveal that PDE9i–psilocybin combination enhances synaptogenesis-related signaling while reducing 5-HT_{2A}R downstream pathways

Proteomic analysis of mPFC synaptosomes collected 24 h 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 PDE9i–psilocybin, including G protein-coupled receptor (GPCR) and serotonin receptor signaling. Remarkably, synaptogenesis, believed to be crucial for psychedelic therapeutic effects [11], was upregulated in the combination group. Kinase enrichment analysis predicted upstream kinases regulated by PDE9i–psilocybin. For upregulated proteins, top kinases were involved in synaptogenesis and structural remodeling including mTOR and Src. For downregulated proteins, top kinases included downstream targets of canonical Gq-coupled 5-HT_{2A}R signaling such as PKC (Prkca and Prkcb). Taken together, these data suggest that PDE9i–psilocybin suppresses 5-HT_{2A}R-mediated downstream signaling while preserving synaptogenesis. Analysis of single-cell RNA-seq data from the Allen Brain Cell Atlas showed co-expression of Htr2a and Pde9a across cortical glutamatergic neuron classes, including pyramidal tract (PT) neurons (Fig. 1I) [10], identifying candidate cell populations in which PDE9 inhibition may modulate 5-HT_{2A}R signaling.

DISCUSSION

These findings demonstrate that pharmacological inhibition of PDE9 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 elevated synaptogenic signaling pathways in the combination group,

consistent with maintained therapeutic potential. The ability to reduce acute 5-HT_{2A}-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-h post-treatment time-point selected for 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 h of dosing and persisting for at least one month—providing translational grounding for the molecular window captured here [12]. It is possible that the increase in cGMP following PDE9 inhibition functions to block psilocybin-induced hyperexcitability and thereby reduce head-twitch responses. A limitation of this study is the male-only chronic stress cohort; future studies should determine whether these findings extend to females, particularly given emerging evidence for sex-dependent responses to psychedelics [13, 14]. A limitation of the HTR as a readout is that it indexes 5-HT_{2A} activation rather than hallucinogenic potential per se: deliriant hallucinogens such as scopolamine and atropine produce perceptual disturbances in humans without eliciting HTR in rodents [15]. The HTR should therefore be interpreted as a proxy for 5-HT_{2A} engagement rather than a direct correlate of subjective perceptual effects. Collectively, these results support the hypothesis that combination approaches targeting intracellular signaling can modulate the acute profile of psychedelics while preserving their therapeutic mechanisms.

DATA AVAILABILITY

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

G.F. and S.J.J. contributed equally to this work. G.F. and S.J.J. designed and performed experiments, analyzed data, and wrote the manuscript. J.R. and A.L.Y. performed experiments. F.S.M. provided the PDE9 inhibitor and contributed to study design. A.C.K. contributed single-cell transcriptomic analyses and edited the manuscript. J.P.D.A. and J.H.K. contributed to data interpretation and edited the manuscript. C.P. and A.P.K. jointly supervised the work, conceived the study, secured funding, and edited the manuscript. All authors approved the final version and agree to be accountable for all aspects of the work.

COMPETING INTERESTS

This work was supported by a research contract from Freedom Biosciences (A.P.K., C.P., G.F.). Drs. Krystal, and Pittenger are co-inventors on a patent application related to psychedelics. Dr. Kaye previously received contracted research funding from Transcend Therapeutics. Drs. Jefferson and Pittenger previously received research funding from Freedom Biosciences. Dr. De Aquino has received research support from Jazz Pharmaceuticals and Ananda Scientific and has served as a paid consultant for Boehringer Ingelheim. In the past three years, Dr. Pittenger has consulted for Biohaven Pharmaceuticals, Freedom Biosciences, Transcend Therapeutics, UCB BioPharma, Mind Therapeutics, Ceruvia Biosciences, F-Prime Capital Partners, and Madison Avenue Partners; has received research support from Biohaven Pharmaceuticals, Freedom Biosciences, and Transcend Therapeutics; owns equity in Alco Therapeutics, Mind Therapeutics, and Lucid/Care; receives royalties from Oxford University Press and UpToDate; and holds pending patents on pathogenic antibodies in pediatric OCD and on novel mechanisms of psychedelic drugs. Dr. Menniti has served as a consultant for Freedom Biosciences, is a co-inventor on patents claiming the use of PDE9 inhibitors in combination with psychedelic drugs, and previously served as the lead biologist at Pfizer for the PDE9 inhibitor research program that developed PF-04447943. He has no current financial interest related to Pfizer or PF-04447943. Dr. Krystal has served as a consultant for Aptinyx, Inc.; Biogen, Idec, MA; Bionomics, Limited (Australia); Boehringer Ingelheim International; Clearmind Medicine, Inc.; Cybin IRL (Ireland Limited Company); Enveric Biosciences; Epiodyne, Inc.; EpiVario, Inc.; Janssen Research & Development; Jazz Pharmaceuticals, Inc.; Otsuka America Pharmaceutical, Inc.; Perception Neuroscience, Inc.; Praxis Precision Medicines, Inc.; Spring Care, Inc.; Sunovion Pharmaceuticals, Inc. Dr. Krystal has served as a scientific advisory board member for: Biohaven Pharmaceuticals; BioXcel Therapeutics, Inc. (Clinical Advisory Board); Cerevel Therapeutics, LLC; Delix Therapeutics, Inc.; Eisai, Inc.; EpiVario, Inc.; Freedom Biosciences, Inc.; Jazz Pharmaceuticals, Inc.; Neumora Therapeutics, Inc.; Neurocrine Biosciences, Inc.; Novartis Pharmaceuticals Corporation; Praxis Precision Medicines, Inc.; PsychoGenics, Inc.; Tempero Bio, Inc.; Terran Biosciences, Inc. In the past 3 years, Dr. Krystal is an inventor on patents licensed by Yale University to Janssen Pharmaceuticals, Biohaven Pharmaceuticals, Spring Health, Freedom Biosciences, and Novartis Pharmaceuticals. Dr. Kwan has been a scientific advisor or consultant for Boehringer Ingelheim, Eli Lilly, Emory Neuroscience, Freedom Biosciences, Otsuka, and Xylo Bio. Dr. Kwan has received research support from Intra-Cellular Therapies. The remaining authors declare no conflict of interest.

ADDITIONAL INFORMATION

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