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Pharmahuasca reduces ROS production and inflammatory gene expression in the brain in a model of PTSD: exploration by RNA sequencing

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Abstract

Post-traumatic stress disorder (PTSD) is associated with cognitive deficits, oxidative stress and inflammation. *N,N*-dimethyltryptamine (DMT) is a known neuroprotective, antioxidant, anti-inflammatory, and psychoplastogen with antidepressant effects. Therefore, we tested the capacity of DMT, the monoamine oxidase inhibitor (MAOI) harmaline, and “pharmahuasca” (DMT + harmaline) to reduce reactive oxygen species (ROS) production and inflammatory gene expression and modulate neuroplasticity-related gene expression in a predator exposure and psychosocial stress rat model of PTSD. We administered DMT (2 mg/kg IP), harmaline (1.5 mg/kg IP), or pharmahuasca every other day for 5 days. We measured ROS production in the prefrontal cortex (PFC) and hippocampus (HC) by electron paramagnetic resonance spectroscopy (EPR) and extracted total RNA from the PFC for sequencing. We also performed *in vitro* assays to measure the affinity and efficacy of DMT and harmaline at the 5HT_{2A}R. DMT and pharmahuasca reduced ROS production in the PFC and HC, while harmaline had mixed effects. RNA sequencing implicated genes related to ROS production, inflammation, neurotransmission, and neuroplasticity. DMT, but not harmaline exhibits both affinity and efficacy at the human 5HT_{2A}R. DMT and pharmahuasca exhibit broad effects that may facilitate the treatment of PTSD by reducing ROS production and inflammatory gene expression, and inducing neuroplasticity.

Introduction

Post-traumatic stress disorder (PTSD) is a trauma and stressor-related psychiatric syndrome characterized by intrusive thoughts and memories, hyperarousal, reexperiencing symptoms, and negative alterations to cognition and mood.¹ PTSD is also linked to psychological and physiological comorbidities, including major depressive disorder (MDD)², anxiety disorders, substance use disorder (SUD),³ type 2 diabetes⁴, cardiovascular disease, inflammatory bowel disease, and chronic kidney disease.⁵ Each of these conditions, including PTSD, is associated with elevated oxidative stress (OXS) and sterile inflammation (INF).⁶ Elevated OXS and INF are thought to arise from PTSD-related chronic stress, and enigmatically, comorbid inflammatory conditions and/or genetics that predispose the individual to reduced antioxidant capacity and increased INF may predispose individuals to developing PTSD.⁶ PTSD is also associated with altered cortical⁷ and hippocampal volume⁸ and cognitive deficits,⁹ which may preexist and play a role in PTSD development.¹⁰⁻¹² Chronic cases of PTSD can lead to neuroprogression in which patients experience further memory impairments and cognitive deficits.⁶ PTSD is therefore bidirectionally associated with altered whole organism allostasis, a characterization shared with major depression (MDD).¹³

In this regard, we have demonstrated that a predator exposure/psychosocial stress (PE/PSS) model of PTSD in rats is associated with elevated central and peripheral INF and reactive oxygen species (ROS) production.¹⁴ The only FDA approved pharmacotherapies for PTSD are the SSRIs sertraline and paroxetine. Our lab demonstrated in this model that sertraline treatment reduced inflammation in the prefrontal cortex (PFC) and hippocampus (HC) and increased 5-HT levels.¹⁵ However, in practice, treatment with SSRIs for PTSD is insufficient^{16,17} and novel efficacious strategies will likely require atypical pharmacology and treatment approaches.^{18,19}

N,N-dimethyltryptamine (DMT), a serotonergic psychedelic 5-HT_{2A}R/5-HT_{1A} agonist, has recently gained attention as an experimental therapeutic for PTSD, MDD, and anxiety disorders.²⁰⁻²⁴ DMT is one component of a traditional plant-based tisane used by indigenous peoples of the Amazon basin. It is commonly called “Ayahuasca” by Westerners and is essentially a combination of two plants: (1) *Psychotria viridis* (Chacruna) containing DMT and (2) *Banisteriopsis cippi* containing the monoamine oxidase inhibitors (MAOIs) harmine (HA), harmaline (HL) and tetrahydroharmaline (THH), which also acts as a weak SSRI.²⁵ Pharmahuasca²⁶ is a pharmaceutical combination of two of the most studied components of Ayahuasca, DMT and HL. DMT is not orally active at standard doses due to its rapid metabolism by monoamine oxidase (MAO) enzymes, but when paired with MAOIs like HL, oral DMT causes an intense psychedelic experience lasting 4 to 6 hours.^{23,27}

Ayahuasca is the most researched formulation of DMT for the treatment of psychiatric illnesses, where it has been studied in Peru, Brazil and Canada.^{20,24,28-32} Further, Ayahuasca treatment in humans is associated with

immunomodulatory effects²⁵ and reduced peripheral blood C-reactive protein levels, which correlate with its antidepressant effects.³³ DMT has also exhibited anti-inflammatory and tissue protective effects *in vitro*³⁴ and in animal models,³⁵ enhancements in hippocampal neurogenesis, a prerequisite for antidepressant effects,³⁶ and has been deemed a psychoplastogen for its ability to induce mTOR-dependent neuroplasticity.³⁷⁻⁴⁰ In addition, all three major β -carbolines in *B. cappi*, HA, HL and THH, also induce neurogenesis *in vitro*.⁴¹ DMT, Ayahuasca⁴² and pharmahuasca may therefore improve cognitive deficits and facilitate psychological processing in PTSD through psychoplastic effects, and normalize physiological homeostasis through tissue protective, antioxidant, and anti-inflammatory effects, reducing whole organism allostatic load.

We therefore hypothesized that DMT and/or pharmahuasca would reduce ROS production and inflammatory gene expression in the PFC and ROS production in the HC of rats in a PE/PSS model. Additionally, we hypothesized that DMT and/or pharmahuasca would induce the expression of genes and pathways associated with neuroplasticity. To test this hypothesis, we utilized a PE/PSS model of PTSD in rats established by Zoladz et al. in Dr. David Diamond's lab and previously behaviorally validated in our lab^{14,15,43-45} and others.^{14,45-48} To determine the capacity of DMT (2 mg/kg IP) alone and in combination with HL (1.5 mg/kg IP) (pharmahuasca) to alter brain reactive oxygen species (ROS) production, we measured ROS production in the PFC and HC by electron paramagnetic resonance spectroscopy (EPR). To examine differential gene expression (DEG), we performed total RNA sequencing on PFC RNA. We performed pathway analysis on DEG data by Gene Ontology (GO), KEGG, and Ingenuity Pathway Analysis (IPA). Finally, based on the RNAseq data, we hypothesized that DMT and HL would both exhibit affinity and efficacy at the human 5HT_{2A}R. To test this, we performed *in vitro* assays to measure the affinity and efficacy of DMT, HL, and 5-HT at the human 5HT_{2A}R (**Fig. 1a**). To our knowledge, we are the first to show that DMT and pharmahuasca reduce ROS production in the PFC and HC. We additionally show that DMT, harmaline and pharmahuasca modulate the expression of genes associated with ROS production, inflammation, and neuroplasticity and that pharmahuasca reduces the expression of genes and pathways associated with neuroinflammation. Finally, we show that 5-HT and DMT, but not harmaline show affinity and efficacy at the human 5-HT_{2A}R.

Results

Oxidative stress analysis

PE/PSS increases ROS production in the PFC and HC, effects that are reversed with DMT and pharmahuasca treatment

We previously demonstrated elevated ROS production in the HC, PFC, and adrenal glands of the PE/PSS rat model of PTSD.¹⁴ In this study, we quantified ROS production in the PFC and HC in control and PE/PSS rats treated with either vehicle, DMT, HL, or pharmahuasca. Compared to control, the PE/PSS group exhibited significantly increased ROS production in the PFC (n=5-7/ group, KW statistic: 24.56, p<.0001) and the HC (n= 5-7/ group, KW statistic: 13.59, p=.0087). DMT reduced ROS in the PFC (mean rank diff = 11, p=.0520, q=.0364) and the HC (mean rank diff = 12.2, p=.0219, q=.0077), and pharmahuasca reduced ROS production in both the PFC (mean rank diff = 15.14, p=.0075, q = .0079) and the HC (mean rank diff = 15.49, p=.0036, q = .0038). HL did not reduce ROS production in the PFC (mean rank diff = 2.429, p= .6680, q = .3507) or the HC (mean rank diff = 2.367, p=.6673, q = .1752) and actually increased ROS production in several animals (**Fig. 1b, c**). HL increased ROS in 3/7 animals in the PFC and 2/6 animals in the HC (**Sup. Fig. 1a, b**).

Figure 1: a) Experimental timeline. Created with BioRender.com **b)** Differences in ROS production in the PFC calculated by the nonparametric Kruskal-Wallis test (24.56, $n=5-7$ / group, $p<.0001$). Multiple comparisons revealed significant differences between Control and PE/PSS PFC ($n=7$, $0.003040 \pm .00018 \mu\text{M/mg protein/ min}$, mean rank = 4.714, $n=5$, $0.012580 \pm .000981 \mu\text{M/mg protein/ min}$, mean rank = 28.00, respectively, mean rank diff = -23.29, $p<.0001$, $q<.0001$), PE/PSS and PE/PSS + DMT ($n=7$, $0.005376 \pm .000558 \mu\text{M/mg protein/ min}$, mean rank = 17, mean rank diff = 11, $p=.0520$, $q=.0364$), and PE/PSS and PE/PSS + pharmahuasca (DMT + Harmaline) ($n=7$, $.004264 \pm .000359 \mu\text{M/mg protein/ min}$, mean rank = 12.86, mean rank diff = 15.14, $p=.0075$, $q=.0079$), but not between PE/PSS and PE/PSS + Harmaline ($n=7$, $.018306 \pm .005556 \mu\text{M/mg protein/ min}$, mean rank = 25.57, mean rank diff = 2.429, $p=.6680$, $q=.3507$) **c)** Differences in ROS production in the HC calculated by the nonparametric Kruskal-Wallis test (13.59, $n=5-7$ / group, $p=.0087$). Multiple comparisons revealed significant differences between Control and PE/PSS ($n=6$, $.005692 \pm .000284 \mu\text{M/mg protein/ min}$, $n=5$, $0.016768 \pm .003941 \mu\text{M/mg protein/ min}$ respectively, mean rank = 12.33, mean rank 2 = 25.20, mean rank diff = -12.87, $p=.0194$, $q=.0077$), between PE/PSS and PE/PSS + DMT ($n=7$, $.007091 \pm .001310 \mu\text{M/mg protein/ min}$, mean rank = 13.00, mean rank diff = 12.2, $p=.0219$, $q=.0077$), PE/PSS + pharmahuasca ($n=7$, $.005785 \pm .000924 \mu\text{M/mg protein/ min}$, mean rank = 9.714, mean rank diff = 15.49, $p=.0036$, $q=.0038$), but not between PE/PSS and PE/PSS + Harmaline ($n=6$, $.020614 \pm .007326 \mu\text{M/mg protein/ min}$, mean rank = 22.83, mean rank diff = 2.367, $p=.6673$, $q=.1752$)

Figure 2: Volcano plot and heatmap for pair wise comparisons between the Control and PE/PSS groups (LFC > 1, FDR > 0.1). b) The heatmap illustrates supervised hierarchical clustering shows of the averaged counts for a subset of genes implicated in PE/PSS pathology.

PE/PSS alters the transcriptome in the PFC

We observed 193 out of 17,573 (1%) total identified genes that significantly differed between PE/PSS and control (FDR < .01) (**Fig. 2**). This genes set comprises genes related to inflammation, growth factor signaling, and neurotransmission.

First, we found an upregulation of interleukin-1 receptor, type I (*Il1r1*) in PE/PSS compared to control. This previously unreported target corresponds with our prior finding of the upregulation of the NLRP3 inflammasome and its product, interleukin 1 beta (IL-1 β), a pro-inflammatory cytokine and ligand of IL-1R1.¹⁴ These data support the fact that neuroinflammatory signaling is elevated in this PE/PSS model.

Second, we observed alterations to growth factor transcription including the upregulation of insulin-like growth factor 2 (*Igf2*) and insulin-like growth factor-binding protein 2 (*Igfbp2*) in PE/PSS compared to control.

Third, we observed an alteration to GABA signaling, an effect also observed in humans with PTSD.⁴⁹ We found a 2.97-fold upregulation of GAT2 (*Slc6a13*) (**Fig. 3a**), a GABA transporter typically expressed at low levels in the brain but that is transcriptionally induced after PE/PSS. Gabrg1, a subunit of the GABA_AR linked to alcoholism⁵⁰ was downregulated in PE/PSS vs control. Somatostatin (*Sst*) was downregulated in PE/PSS, as has been observed in post mortem dlPFC brain tissue from PTSD patients.⁴⁹ Together, these findings may indicate a shift in the excitatory/inhibitory balance in the PFC.

DMT alters the transcriptome in the PFC of PE/PSS rats and reverses some molecular changes observed between control and PE/PSS animals

DMT treatment in PE/PSS rats altered the expression of 3869 identified genes out of 17,802 total genes measured in this comparison, or 22% (**Sup. Fig. 4**). We observed that PE/PSS animals exposed to three doses of DMT (2mg/kg IP) exhibited reduced expression of genes involved in oxidative phosphorylation in the mitochondria. GO and KEGG analysis support the downregulation of mitochondrial pathways (**Sup. Fig 7a, 8a**). DMT modulated the NRF2-Mediated Oxidative Stress Response IPA pathway (**Sup. Fig. 9**). DMT downregulated *Nrf2* and upregulated *Keap1*, its molecular inhibitor. The transcriptional changes observed herein confirm that DMT treatment alleviates oxidative stress in PE/PSS.

DMT reversed PE/PSS-induced changes to GAT2, increased the expression of *Slc32a1*, a vesicular GABA transporter downregulated in human PTSD.⁴⁹ It upregulated *Gabbr1*, *Gabbr2*, subunits of the G_i coupled GABA_B receptors that mediate the effects of gamma-hydroxybutyric acid (GHB), upregulated *Gabrb1*, reduced *Gabrg1* and *Gabrg2* and reduced *Gabra1* and *Gabra2*, subunits of the GABA_AR that are upregulated in human PTSD.⁴⁹ DMT upregulated GAD1 and GAD2, enzymes that metabolize glutamate to GABA, the latter of which is reduced in the dlPFC in human PTSD.⁴⁹ DMT increased the expression of *Aldh9a1*, an enzyme that metabolizes GABA into GHB, and *Aldh5a1*, a mitochondrial enzyme that metabolizes GABA into succinate, an input into the citrate cycle (**Fig. 3, Fig. 6a**). DMT reversed PE/PSS induced changes to *Sst* (**Sup. Fig. 3j**) and upregulated its receptors *Sstr1*, *Sstr2*, and *Sstr4*.

DMT upregulated genes associated with glutamatergic neurotransmission (**Fig. 6b**) and neuroplasticity including the presynaptic genes synapsin (*Syn2*, *Syn3*) and synaptotagmin (*Syt1*), the postsynaptic genes for NMDAR (*Grin1*), AMPA (*Gria1*), ephrin-B (*Efnb2*) and its receptor, EPHB (*Ephb3*, *Ephb6*). DMT upregulated *Lrp8* and growth factor receptors (GFR), like *Ntrk2* and *Ntrk3* and downstream effectors responsible for new protein synthesis including mTORC1, mTORC2 (**Fig. 7**), and CREB binding protein (CBP/*Crebbp*). DMT downregulated *Il1r1*, *Anxa2*, *Vtn* and *Igf2* (**Sup. Fig. 3**), which were upregulated in PE/PSS animals, and modulated the expression of many cytokines and pattern recognition receptors (PRR) (**Fig. 6d**). DMT significantly altered the expression of G proteins (G_{αs}, G_{αi}, and G_{0αq}) (**Sup. Fig. 15**), G protein-coupled receptors (GPCRs) including 5-HT_{1A}R (*Htr1a*) and 5-HT_{1B}R (*Htr1b*) (**Fig. 6c**), among others, and their effectors.

a) Control vs PE/PSS

b) PE/PSS vs DMT

Figure 3: Ingenuity pathway analysis schematic of transcriptional changes in the IPA GABA receptor signaling pathway. **a)** PE/PSS Vehicle vs Control animals. **b)** PE/PSS + DMT vs PE/PSS + Vehicle. Red/Green indicate up/down regulated genes respectively, grey indicates uncertainty in the direction of abundance and white indicates parts of the pathway that do not significantly differ between groups.

HL alters the transcriptome in the PFC of PE/PSS rats and reverses some molecular changes observed between control and PE/PSS animals

Surprisingly, HL elicited the greatest total number of significant transcriptional changes compared to PE/PSS + vehicle (5279/17769 or 30%) (**Sup. Fig. 5**). This large transcriptional effect should be considered cautiously because several animals treated with HL (**Fig. 1b, c**) exhibited increased ROS production in the PFC and HC, which did not occur in any animal treated with DMT or pharmahuasca. HL upregulated the IPA Synaptogenesis Pathway, indicating neuroplasticity-inducing effects. HL had the most substantial effects on the ephrin system, modulating both EphA and EphB and their ligands EfnA and EfnB. HL modulated G proteins (G_{α_s} , G_{α_i} , and G_{α_q}) (**Sup. Fig. 11**) and various GPCRs and their effectors. HL modulated the expression of over a dozen genes involved in mitochondrial oxidative phosphorylation including downregulating ND1, ND2, ND3, ND4, ND5, COX1, COX2, COX3 and upregulating ATP5D and ATP5A1, among others and modulated GO, KEGG and IPA mitochondrial pathways (**Fig. 4a, Sup. Fig 7c, 8b**). HL upregulated *Sst*, *Sstr1*, *Sstr2*, *Sstr3* and *Sstr4*.

a) PE/PSS vs Harmaline

b) PE/PSS vs pharmahuasca

Figure 4: a) Effects of PE/PSS + Harmaline vs PE/PSS + Vehicle on the IPA Oxidative Phosphorylation signaling pathway. **b)** Effects of PE/PSS + pharmahuasca vs PE/PSS + Vehicle on the IPA Oxidative Phosphorylation signaling pathway. Red/Green indicate up/down regulated genes respectively, grey indicates uncertainty in the direction of abundance and white indicates parts of the pathway that do not significantly differ between groups.

Pharmahuasca alters the transcriptome in the prefrontal cortex of PE/PSS rats and reverses some molecular changes observed between control and PE/PSS animals

The effects of pharmahuasca were similar to those of each component alone but were also unique in several ways (Sup. Fig 2). Pharmahuasca significantly modulated the expression of 2,969 genes out of 17690 detected (17%) (Sup. Fig. 6). Like the other treatments, pharmahuasca increased the expression of the *Igf2r*, had the greatest effect on *Igf2*, and was the only treatment to normalize *Igf2* levels (Sup. Fig 3). Pharmahuasca was the only treatment to downregulate NF-κB2 (*Nfkb2*) (Fig. 5, Sup. Fig. 3e) and, like the other treatments, downregulated numerous cytokines and PRRs (Fig. 5, 6d). These effects are supported by the downregulation

of the GO Biological Process Pathways for Innate Immunity (**Sup. Fig. 7f**) and the IPA Pathway for Neuroinflammation (**Sup. Fig. 12**). Pharmahuasca was the most efficacious at reducing ROS and downregulated the IPA Oxidative Phosphorylation signaling pathway, including numerous mitochondrial genes and unlike HL, did not upregulate any mitochondrial genes (**Fig. 4b**). Pharmahuasca downregulated *gp91phox*, *p22phox*, *p47phox*, and *Rap1*, functional subunits of the NADPH oxidase enzyme involved in ROS production by macrophages and microglia and downregulated the IPA Role of Nitric Oxide and ROS in Macrophages signaling pathway (**Fig. 5**). Pharmahuasca, like DMT and HL, downregulated *Nrf2* and upregulated *Keap1* (**Sup. Fig. 9**). Pharmahuasca upregulated the IPA Synaptogenesis Pathway, indicating the induction of neuroplasticity (**Sup. Fig. 13**). Pharmahuasca upregulated the IPA Opioid Signaling Pathway and was the only treatment to upregulate both the delta opioid receptor (*Oprd1*) and the receptor for nociception (*Oprl1*) (**Sup. Fig. 14**). Pharmahuasca upregulated mGluR5 (*Grm5*) among several other glutamatergic targets (**Fig. 6b**). Pharmahuasca altered the expression of transcripts involved in GABA signaling (**Fig. 6a**), ephrin signaling (**Sup. Fig. 13**), $G\alpha_q$, $G\alpha_i$, and $G\alpha_q$ linked GPCRs and their effectors, insulin receptor signaling, and like DMT, mTORC1 and mTORC2 signaling (**Fig. 7**). Pharmahuasca modulated the IPA pathway for CREB signaling (**Sup. Fig. 15**) and was the only treatment to reduce the expression of Indolethylamine-N-methyltransferase (*Inmt*) (**Sup. Fig. 3o**). Pharmahuasca upregulated *Sstr1*, *Sstr3*, and *Sstr4*.

HL does not exhibit affinity or efficacy at the human 5-HT_{2A}R in HEK cells

Based on the high degree of overlap in the transcriptional effects of DMT and HL, particularly on genes related to neuroplasticity, we next sought to determine if, like DMT, HL exhibits appreciable affinity or efficacy at the 5-HT_{2A}R. HL did not exhibit substantial binding ($K_i > 10,000$ nM) or calcium flux (~7% 5-HT E_{MAX}) downstream of 5-HT_{2A}R activation. On the other hand, the K_i of DMT for the human 5-HT_{2A}R was 511 nM and the E_{MAX} & EC_{50} for activating calcium flux were 95% 5-HT and 19 nM, respectively (**Fig. 6e, f, Sup. Fig. 17**).

K_i (nM) $\pm$ SEM

Drug	h5-HT _{2A}	r5-HT _{2A}	h5-HT _{1A}	r5-HT _{1A}
	[³ H]ketanserin	[³ H]ketanserin	[³ H]8OH-DPAT	[³ H]8OH-DPAT
5-HT	187 $\pm$ 51	614 $\pm$ 43 ⁶¹	2.67 $\pm$ 0.33 ⁶³	2.2 $\pm$ 0.5 ⁶⁴
DMT	511 $\pm$ 67	660 $\pm$ 30 ⁶²	450 $\pm$ 150 ⁶³	200 $\pm$ 10 ⁶²
Harmaline	> 10,000	7790 $\pm$ 1200 ⁶³	ND	> 10,000 ⁶²

f)

	DMT	Harmaline	5-HT
Bottom	= 0.000	= 0.000	= 0.000
Top	95.58	7.754	100.0
LogEC50	-7.702	-7.265	-9.209
EC50	1.986e-008	5.435e-008	6.187e-010
Span	= 95.58	= 7.754	= 100.0

Figure 6: (a-f) Effects of all three treatments on GABA, Glutamate, and cytokines and pattern recognition receptors graphed across all groups. **a)** GABA receptors and transporters graphed across all groups. **b)** Glutamate receptors and transporters graphed across all groups **c)** Serotonin receptors graphed across all groups **d)** Cytokines and pattern recognition receptors graphed across all group **e)** Binding affinities for 5-HT, DMT, and harmaline at human and rat 5-HT_{2A} and 5-HT_{1A} (ND: No data available). **f)** Comparison of DMT and harmaline relative to serotonin (5-HT) in the human 5-HT_{2A} Gq-dependent calcium flux assay

Discussion

We report that [1] PE/PSS is associated with elevated ROS production and inflammatory gene transcription (*Il1r1*), confirmation of our previous work¹⁴, and novel changes to growth factor expression (*Igf2*, *Igf2r*) and GABAergic neurotransmission (*Slc6a13*, *Gabrg1*, *Sst*). [2] Pharmahuasca and DMT reduced ROS production in the PFC and HC and HL exhibited highly variable effects on ROS production. [3] All treatments altered the NRF2 pathway and mitochondrial oxidative phosphorylation pathways. Pharmahuasca reduced transcription of the NADPH oxidase pathway. [4] All treatments reduced *Il1r1* transcription in addition to numerous other inflammatory cytokines. Pharmahuasca downregulated NF- κ B2 (*Nfkb2*), a transcription factor for inflammatory cytokines. Pharmahuasca reduced the GO Innate Immune Pathway and the IPA Neuroinflammation Pathway, confirming that pharmahuasca reduced the transcription of inflammatory pathways. [5] All treatments downregulated *Igf2* and upregulated *Igf2r*. Pharmahuasca normalized *Igf2r*. [6] All treatments normalized GAT2 (*Slc6a13*) and modulated numerous other GABA signaling related transcripts. DMT normalized somatostatin (*Sst*) and upregulated its receptors *Sstr1*, *Sstr2* and *Sstr4*. [7] All treatments upregulated genes associated with neuroplasticity; DMT and pharmahuasca upregulated mTOR signaling, and pharmahuasca upregulated the CREB pathway. [8] DMT and 5-HT, but not HL bind to the 5-HT_{2A}R and provoke Ca²⁺ release in HEK cells.

Based on previous work¹⁴ and our initial data (**Sup. Fig. 1a,b**), we hypothesized that PE/PSS animals would exhibit a transcriptional signature of elevated ROS that would be attenuated by treatment, but we did not observe any direct transcriptional links to elevated ROS production between PE/PSS and control. Therefore, the mechanism underpinning the increase in ROS in PE/PSS is likely functional and/or post-transcriptional. Increased ROS production may result from elevated neuroimmune signaling, which is supported by this study (*Il1r1*) and our previous work (NALP3, IL-1 β , TLR4).^{14,45} Elevated ROS in PE/PSS could also emerge from the disinhibition of prefrontal networks caused by altered GABAergic signaling (upregulation of GAT, downregulation of *Sst*). Increased *Igf2* signaling, reduced *Igf1*, increased norepinephrine, decreased serotonin, and/or increased levels of corticosterone observed previously could also contribute.^{14,43,45} Collectively, these effects could lead to elevated neural activity and subsequently to elevated mitochondrial activity without necessarily leading to increased mitochondria and a measurable transcriptional effect.

Effects on ROS pathways were clearer in treated animals and we have elucidated three mechanisms by which these treatments could modify ROS production. [1] All three treatments altered the IPA NRF2-Mediated Oxidative Stress Response pathway, downregulating *Nrf2* and upregulating its modulator and ROS sensor, *Keap1*. *Keap1* senses oxidative stress through cysteine residues. In response to stress, a molecular mechanism within *Keap1* allows *Nrf2* to escape ubiquitination and translocate to the nucleus where it activates antioxidant responses (**Sup. Fig. 9**). [2] Pharmahuasca had the greatest effect on ROS and the IPA Production of Nitric Oxide and ROS in Macrophages pathway, leading to the downregulation of CYBA (*p22phox*), CYBB (*gp91phox*), *p47phox*, and *Rap1*, all components of NADPH oxidase 2 (NOX2) (**Fig. 5**). NOX2 produces superoxide in microglia and is known to exacerbate traumatic brain injury.⁵¹ DMT did not impact either CYBA or CYBB, HL only reduced CYBB expression, and all three treatments modulated the expression of *Rap1*. Importantly, these data suggest a special effect of pharmahuasca. [3] All three treatments reduced the expression of mitochondrial transcripts, as evidenced by DMT and HL downregulating pathways for respiratory chain and NADH dehydrogenase (**Sup. Fig. 7a, c, 8a, b**) and both HL and pharmahuasca downregulating the IPA Oxidative Phosphorylation Pathway (**Fig. 4**), albeit somewhat differently. HL downregulated components of complex I, the greatest source of mitochondrial ROS, and complexes III, IV and V, but also upregulated genes in each. Pharmahuasca downregulated many of the genes that HL did, in addition to others, but did not upregulate any mitochondrial transcripts. Together, these data support a multifaceted interpretation of the effects of DMT, HL, and pharmahuasca on brain ROS production with all three treatments impacting the three pathways differently.

It is well understood that there exists a reciprocal relationship between ROS and inflammation such that ROS can lead to OXS, leading to cell damage, activation of DAMPS, and subsequent inflammation. Inflammation

can then lead to the production of additional ROS, (superoxide excreted from macrophages and microglia) through NADPH oxidase 2.^{52,53}

PE/PSS was associated with increased expression of *Il1r1*, the receptor for IL-1 β , a pro-inflammatory cytokine that we previously established is increased in the PFC in this model. *Il1r1* was significantly reduced by all three treatments, indicating the anti-inflammatory effect of these compounds. Treatments reduced expression of *Il1 α* , *Tlr4*, *Tlr6*, *Tlr7*, *Ifngr1*, and a host of different cytokines to different extents, depending on the treatment (**Fig. 6d**). However, only pharmahuasca significantly reduced the expression of NF- κ B2 (*Nfkb2*), a functional analogue of NF- κ B, which was reduced by 5-methoxy-DMT in human brain organoids.⁵⁴ In accord with this, only pharmahuasca downregulated the GO Biological Process Innate Immunity Pathway (**Sup. Fig. 7f**) and the IPA Neuroinflammation Pathway (**Sup. Fig. 12**). Together, these results suggest that DMT and HL have modest anti-inflammatory effects in the brain at the dosing regimen implemented here, but stronger effects were observed in combination as pharmahuasca. This may be a result of the short half-life of DMT in the rat brain ($6.5 \pm .7$ minutes at 3.2 mg/kg – $12.5 \pm .9$ minutes at 10 mg/kg),⁵⁵ that is extended in pharmahuasca through MAO inhibition by HL.

It is noteworthy however that in a rat model of allergic asthma, DMT and 5-methoxy-DMT did not exhibit anti-inflammatory or protective effects, while other psychedelic 5-HT_{2A}R agonist, including tryptamine derivatives, like psilocin (4-HO-DMT), did. Yet, both DMT compounds were given intranasally at low doses (0.5 mg/kg), without an MAOI.⁵⁶ All other existing studies in the DMT literature used, at minimum, 1 mg/kg IP as a “microdose” and up to 10 mg/kg as a “high dose.” The asthma study (.5 mg/kg IN), this work (2 mg/kg IP), and recent work studying repeated so-called “microdoses” of DMT (1 mg/kg IP), all gave multiple doses over time.³⁷⁻⁴⁰ The primary metabolic enzyme for DMT and 5-MEO-DMT, MAO is present in the nasal mucosa⁵⁷ and in conjunction with the low dose, may have prevented sufficient DMT from reaching the lung. Furthermore, the anti-inflammatory and tissue protective activity of DMT may be mediated by the Sigma-1 receptor⁵⁸, which exhibits reduced expression in alveolar cells compared to neurons.⁵⁹ While interesting, the asthma study does not represent conclusive evidence against the anti-inflammatory activity of DMT in all tissues, and at all doses, dosing regimens, and experimental conditions.

We observed increased expression of *Igf2* and *Igfbp2* mRNA in the PFC of PE/PSS animals compared to control. *Igf2* is a paternally linked gene that may play a role in the intergenerational effects of trauma and the effects of maternal stress on offspring.⁶⁰⁻⁶² IGF2 may mediate memory deficits and HPA axis alterations across numerous psychiatric diseases including PTSD.^{63,64} The literature on IGF2 in the brain is focused on the HC, where acute stress sharply increases IGF2 expression and mediates fear memory acquisition,⁶⁵ but chronic stress reduces it and it mediates depressive behaviors.⁶⁶ It is unknown how chronic stress impacts IGF2 expression in the PFC. IGF2 is produced by the leptomeninges, endothelial cells, choroid plexus, stem cells, and progenitor cells of the hippocampal subgranular zone (SGZ), and bathes the brain through the ventricular system.^{67,68} Typically, astrocytes, microglia, and neurons do not express IGF2 mRNA, but it can be induced in human astrocytes and microglia by IL-1 β and LPS, respectively.⁶⁹ Additionally, it is induced by oxidized low density lipoprotein (OX-LDL) in macrophages.⁷⁰ This PE/PSS model is characterized by chronically elevated IL-1 β and ROS, which can lead to oxidized lipid species. IL-1 β and ROS are therefore excellent candidates for the induction of IGF2 expression. DMT, HL, and pharmahuasca reduced *Igf2* transcription and increased transcription of the *Igf2r*, *Insr*, and *Igf1r*. Pharmahuasca had the greatest effects on ROS production, innate immune pathways, and *Igf2*, and is the only treatment to normalize *Igfbp2* expression (**Sup. Fig. 3c**). Taken together, it is plausible that the elevated *Igf2* expression in this model is linked to higher baseline ROS and IL-1 signaling and that these treatments attenuate ectopic and/or elevated *Igf2* expression through the modulation of these factors.

Psychedelics, including DMT, induce 5-HT_{2A}R-dependent neuroplasticity, comparable to the recently FDA-approved antidepressant NMDAR antagonist, ketamine.³⁷⁻⁴⁰ *In vitro* experiments with lysergic acid diethylamide (LSD) and ketamine suggest that there are two requisite phases for the induction of neuroplasticity, an initial “stimulation” phase and a secondary “growth” phase.⁷¹ Existing evidence suggests that serotonergic psychedelics activate the 5-HT_{2A}R through unique functional selectivity, which leads to the selective recruitment of

GPCR effectors (**Sup. Fig. 10**). This leads to increased AMPAR (*Gria1*) activation, likely through elevated calcium signaling downstream of Gα_q, triggering brain-derived neurotrophic factor (BDNF) release and the initial induction of a plastic state through TrkB receptors, the stimulation phase. Subsequently, mTOR complex 1 and/or 2 (mTORC1, mTORC2) (**Fig. 7**) are activated, leading to CREB activation^{72,73} (**Sup. Fig. 15**) and the transcription of genes required for synapse formation, including ephrin and its receptors EphB and EphA, the growth phase (**Sup. Fig. 13**). We observed a similar molecular pattern after all three treatments in the current study. Collectively, these factors underpin synaptogenesis and long term potentiation (LTP), critical mechanisms for cognitive flexibility and learning (**Fig. 8**).

Figure 8: Effects of PE/PSS + Pharmahuasca vs PE/PSS + Vehicle on the IPA Long Term Potentiation (LTP) pathway. Red/Green indicate up/down regulated genes respectively, grey indicates uncertainty in the direction of abundance and white indicates parts of the pathway that do not significantly differ between groups.

Finally, we hypothesized that the overlapping effects of both compounds are mediated through agonist activity at the 5-HT_{2A}R. To test this, we used a surrogate cellular model to determine the binding affinity and Ca²⁺ signaling pathway activation at the human 5-HT_{2A}R for DMT and HL. We confirmed the null hypothesis that HL does not exhibit substantial binding affinity (**Fig. 6e, Sup. Fig. 17**) or functional activity at the human 5-HT_{2A}R (**Fig. 6f**). These data suggest that the observed overlap in the transcriptional effects is convergent through 5-HT_{2A}R agonism and MAOI activity, rather than direct activity at the 5-HT_{2A}R by HL.⁷⁴ However, HL likely increased the concentration of 5-HT, which may have acted through the 5-HT_{2A}R to induce the overlapping effects. In fact, both HL and DMT upregulated the IPA G_q pathway (**Sup. Fig. 10, 11**), but HL altered the expression of more genes overall than DMT. HL may therefore exert effects on the terminal domains of broad neuromodulator systems (MAOI), compared to the selective effects of 5-HT_{2A}R/5-HT_{1A}R agonism (**Fig. 6f**).⁷⁵⁻⁷⁸ These data are not conclusive however; cell types and biomolecular context (i.e., effector expression, membrane lipid composition, receptor ensemble energy landscape) in which the competition binding and signaling assays were performed may impact these values. It is possible that DMT and HL bind with altered affinity and/or produce distinct functional changes in a state of chronic inflammation, oxidative stress, or at a different local pH compared to a “normal” individual.⁷⁹

Taken together, this work highlights the capacity of 5-HT_{2A}R agonist psychedelics, like DMT and pharmahuasca to reduce allostatic load and facilitate recovery from traumatic stress through four mechanisms: [1] reducing ROS production, [2] reducing inflammation, [3] modulating growth factors and neurotransmitter signaling, and subsequently [4] modulating neuroplasticity. Each of these effects could be driven by 5-HT_{2A}R agonism,⁸⁰⁻⁸³ but each could also be enhanced by concurrent sigma-1 receptor agonism (**Sup. Fig. 20**).^{28,34,84,85}

The purpose of this study was to examine biological endpoints related to PE/PSS. Therefore, a major limitation of this work is that it was beyond the scope to investigate the PTSD-like behaviors previously measured in our lab^{14,45} and others;^{46-48,86} however some have already been examined for DMT in rodents^{37,39} and Ayahuasca in humans.^{20,24,29-31} It was also beyond our scope to investigate protein expression and cell type specific changes in brain mRNA expression. The former should be addressed by LC-MS based proteomics paired with immunohistochemistry for specific targets and the latter can theoretically be addressed in our data set using computational methods, given access to single cell sequencing data from the PFC, or by single cell RNA sequencing. Disentangling the exact mechanisms of action of these compounds will require the replication of this work utilizing 5-HT_{2A}R, 5-HT_{1A}R, and sigma-1R selective antagonists, ideally including stress naïve animals to determine if the effects differ between PE/PSS and naïve animals.

Materials and Methods:

Ethics statement: This study was performed in strict accordance with the recommendations of the Institute for Laboratory Animal Research’s 2011 Guide for the Care and Use of Laboratory Animals, under the auspices of an animal care and use protocol approved by the Louisiana State University Institutional Animal Care and Use Committee (15-061). All animal experiments were performed according to ARRIVE guidelines.

Animals

Naïve, adult male Sprague Dawley rats were used in this experiment. Rats were bred in the LSU School of Veterinary Medicine vivarium and were between 10 and 12 weeks of age when the study was initiated. Rats were pair-housed in standard plastic microisolator cages and had access to food and water ad libitum. The cages were maintained in ventilated racks (8X5) and each cage was assigned a rack location at random to ensure even distribution of rack location. The vivarium room was kept on a 12 hour light/dark cycle (0700-1900); room temperature was maintained at 20 ± 1 °C and humidity ranged from 23% to 42%. Predator exposures were performed using two cats, one male and one female (age 9, Harlan Laboratories, Indianapolis, IN, and age 12, Tulane University, New Orleans, LA, respectively). Cats were housed in an open room maintained at the same light/dark cycle, temperature, and humidity as the rat room. This experiment was initially performed and subsequently replicated in two independent cohorts of animals.

Stress induction

Animals were pseudo-randomly assigned into either “control,” “PE/PSS,” “PE/PSS + DMT,” “PE/PSS + HL,” and “pharmahuasca” (PE/PSS + HL + DMT) such that animal date of birth was balanced between groups (n=5-7/ group). The following day, all PE/PSS rats began the predator exposure and psychosocial stress regimen, first published and validated by Zoladz, et al., designed to produce a pre-clinical PTSD-like phenotype that closely mimics signs and symptoms of human PTSD patients.^{46-48,86} PE/PSS induction were performed as previously described.¹⁴

Drug treatment

31 days post predator exposure and psychosocial stress procedure, animals were treated with DMT (2 mg/kg body weight IP), HL (1.5 mg/kg body weight IP), or DMT + HL (2 mg/kg and 1.5 mg/kg IP, respectively) every other day for a total of 3 doses. Drug dosages were selected based on existing literature and a preliminary study testing two doses of DMT, 2 mg/kg and 4 mg/kg IP (**Sup. Fig. 1a,b**). 2 mg/kg DMT was the optimal dose to normalize free radical production in PE/PSS animals. The dose of HL was chosen with the primary objective of ensuring sufficient MAO-inhibition. DMT (Cat.# SML0791, Lot# 083M4613V, ≥97% HPLC, Origin: India) and HL (Cat.# 51330; Lot# BCBK5380V, ≥95%) were purchased from Sigma Aldrich for the preliminary cohort. For the second cohort, DMT was purchased from Cayman Chemical (Cat.# 13959; Lot# 04647877, Origin: MI, USA). DMT was solubilized in 100% ethyl alcohol using a table-top vortex and diluted with sterile saline such that final ETOH concentrations were below 8%, the acceptable level for use as a vehicle. After stress induction, PE/PSS, DMT, HL, and pharmahuasca (DMT + HL) animals were interperitoneally injected first with HL (1.5 mg/kg IP) or vehicle and immediately afterwards with DMT (2 mg/kg IP) or vehicle, as applicable. PE/PSS, DMT, and HL animals were injected with vehicle on the same schedule as animals receiving two drug injections. Initially, we planned to compare these data with orally gavaged Ayahuasca from our collaborator, Dr. Jordi Riba, to study the differences caused by route of administration and additional components of the traditional Ayahuasca brew. While no animal exhibited any concerning physiological side effects, they exhibited behavioral signs of distress and were extremely resistant to taking a second dose of Ayahuasca. These animals chewed through the plastic gavage tube, a behavior we have never before witnessed. Based on these effects, we determined that it was not ethical to continue studying the oral administration of whole plant-derived Ayahuasca in rats.

Electron Paramagnetic Resonance Spectroscopy:

ROS were measured in fresh brain tissue (HC and PFC) as previously published by our lab¹⁴. Briefly, the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH) was used to measure tissue ROS levels. Chopped and hand homogenized pieces of tissue were incubated at 37 °C with CMH (200 μM) for 30 minutes prior to EPR measurement. Aliquots of incubated probe media were then taken in 50 ul disposable glass capillary tubes (Noxygen Science Transfer and Diagnostics) for determination of ROS production. EPR measurements were performed using an EMX ESR eScan BenchTop spectrometer and super high quality factor microwave cavity (Bruker Company, Germany). EPR measurements were normalized by total sample protein using a Pierce BCA Protein Assay Kit (catalog number: 23225).

As this experiment was performed in two separate cohorts with each group equally represented within each cohort, as expected, small but significant differences in baseline levels of ROS production in control groups were observed between the two cohorts; therefore, data were normalized between cohorts to control animals such that both groups could be considered together for overall statistical analysis. The overall pattern of ROS induction in PE/PSS and attenuation by drug treatments were comparable between groups in all cases with the exception of several animals exhibiting increased ROS production in the HL only group (**Sup. Fig. 1**).

RNA sequencing

Whole PFC tissue was placed in Trizol and stored at -80 °C until RNA extraction. Total RNA was extracted using a Qiagen Lipid Tissue Mini Kit Cat. No. 74804, and two aliquots were prepared, one for RNA sequencing, and one for real time RT-PCR confirmation of those data. RNA quality and quantity were measured by Nanodrop Spectrophotometer ND-1000 UV/Vis and by nucleotide fragment analysis on an

Advanced Analytical Fragment Analyzer. RNA quality number in all samples was ≥ 6.8 . 3-4 samples per group were chosen based on optimal RNA quality and shipped to Novogene corporation at UC Davis for RNA sequencing on the Illumina platform. cDNA library construction was performed with 1 μ g of RNA using NEBNext® Ultra 2 RNA Library Prep Kit for Illumina® (cat NEB #E7775, New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol. Novogene enriched the mRNA using oligo (dT) beads and followed this with two rounds of purification and fragmented randomly by adding fragmentation buffer. The first strand of cDNA was synthesized using random hexamers primer. Next, dNTPs, RNase H, DNA polymerase 1, and a custom second strand synthesis buffer (Illumina) were added to produce the second strand (ds-cDNA). Subsequently, terminal repair, poly-adenylation and sequencing adaptor ligation were performed, followed by size selection and PCR enrichment. This resulted in 250-350 bp insert libraries, which were quantified using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Walkham, MA, USA) and quantitative PCR. An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used to determine size distribution. Sequencing was performed on an Illumina NovaSeq 6000 Platform (Illumina, San Diego, CA, USA) using a paired-end 150 run (2x150 bases) Q30 $\geq$ 80% at 20M raw reads/sample. Alignment was performed using hisat2, v2.1.

Real-Time PCR analysis

Semi-quantitative real-time RT-PCR (n=4/group) was used to confirm the mRNA levels of a small subset of significantly differentially expressed genes elucidated by RNA sequencing in the PFC (*Igf2*, *Igf2bp2*, *Slc6a13*, *Clip3*). Very little PFC tissue sample remained after aliquoting tissue samples for EPR and RNAseq, but we chose to confirm this small set of genes based on their contribution to the PE/PSS phenotype and their additional modulation by one or more treatments. All 4 genes were run on at least 3 replicates across 2 PCR runs, totaling 6 runs/ gene/ sample. Total RNA extraction was performed as described above. cDNA synthesis was performed using a Bio-Rad iScript cDNA synthesis kit (Catalog # 1708891) as previously described.⁸⁷ Gene expression was calculated by $\Delta\Delta$ CT and was normalized to GAPDH mRNA levels. These data are presented as fold change of the gene of interest relative to control animals (**Sup. Fig. 16a-d**). Table of primers (**Sup. Fig. 18**). 2/4 measured genes showed the similar differential expression between PCR and RNAseq (**Sup. Fig. 3a, i, 16a, d**). 2/4 measured genes showed higher variability in PCR than RNAseq, but showed the same pattern of expression between groups (**Sup. Fig. 3c, n, 16b, e**), overall confirming the RNAseq data.

Cell Culture Methods

Human embryonic kidney (HEK) 293 cells stably expressing the human 5-HT_{2A} receptor (HEK-h2A; $B_{\max}$ = 1600 fmol/mg protein) were a kind gift from Dr. David Nichols (University of North Carolina, Chapel Hill).⁸⁸ These cells were routinely cultured and maintained in DMEM (Gibco, Cat.# 11054-020) supplemented with 10% (v/v) fetal bovine serum (Gibco, Cat.# 16000-044; Lot# 2216190RP), 2 mM GlutaMAX™, 100 units/mL penicillin, 100 μ g/mL streptomycin, and 100 μ g/mL Zeocin™ within an incubator controlled at 37 °C, 95% humidity and 5% CO₂.

Calcium Flux Assay

G α_q -mediated calcium flux downstream of 5-HT_{2A} receptor activation was determined using the HEK-h2A cell line. Cells were seeded in DMEM (Gibco, Cat.# 11054-020) supplemented with 10% (v/v) fetal bovine serum (Gibco, Cat.# 16000-044; Lot# 2216190RP), 2 mM GlutaMAX™, 100 units/mL penicillin, 100 μ g/mL streptomycin, and 100 μ g/mL Zeocin™ onto 96-well Poly-D-Lysine plates with clear bottoms (12,000 cells/well) and cultured at 37 °C, 95% humidity and 5% CO₂. 48 hours following plating, media was aspirated and replaced with DMEM supplemented with 1% dialyzed fetal bovine serum, 2mM GlutaMAX™, and 100 μ g/mL Zeocin for an additional 12 hour incubation at 37 °C, 95% humidity and 5% CO₂. On the day of the experiment, cells were washed once with HBSS supplemented with 20 mM HEPES, loaded with 75 μ L of 3 μ M Fluo-2 AM HA (Ion Indicators, LLC) diluted in HBSS–HEPES buffer, incubated for 1 hour at 37 °C, 95% humidity and 5% CO₂, washed again with HBSS–HEPES, and maintained in 50 μ L HBSS–HEPES at 25 °C.

The plates of dye-loaded cells were placed into a FlexStation 3 microplate reader (Molecular Devices, LLC) to monitor fluorescence (excitation, 485 nm; emission, 525 nm; cutoff, 515 nm). Plates were read for 20 s (bottom read, 2 s interval, 12 reads/well, PMT: medium) to establish baseline fluorescence and then challenged with DMT, HL, or 5-HT diluted in a range of 10 pM to 100 μ M or buffer and read for an additional 80 s. Each concentration point was tested in triplicate. After obtaining a calcium mobilization trace for each sample, the peak fluorescence in each well minus mean baseline fluorescence (ΔF) was divided by mean baseline fluorescence (F) to give $\Delta F/F$. Data were normalized to the maximum peak fold-over-basal fluorescence produced by 5-HT (100%) and baseline fluorescence (0%) and analyzed using nonlinear regression curve-fitting routines in GraphPad Prism 9.0 (GraphPad Software, Inc.). HL freebase was dissolved in DMSO and the final concentration of DMSO in assay plate was $\leq 1\%$.

Competition Binding Assay

HEK-h2A cells were subcultured and grown in DMEM (Gibco, Cat.# 11054-020) supplemented with 10% (v/v) fetal bovine serum (Gibco, Cat.# 16000-044; Lot# 2216190RP), 2 mM GlutaMAXTM, 100 units/mL penicillin, 100 μ g/mL streptomycin, and 100 μ g/mL ZeocinTM until 90% confluent. The cells were then serum-starved in DMEM (Gibco, Cat.# 11054-020) supplemented with 2 mM GlutaMAXTM, 100 units/mL penicillin, 100 μ g/mL streptomycin, and 100 μ g/mL ZeocinTM for 12 hours. Membranes were prepared by scraping and homogenizing on ice and centrifuged at 2,000 $\times g$ for 10 minutes at 4 $^{\circ}$ C. Membranes were resuspended in cold binding buffer (50 mM Tris-HCl, 10 mM MgCl₂, 0.1 mM EDTA, pH 7.4), homogenized, and centrifuged again. After resuspension in cold binding buffer, 1 mL aliquots were distributed to pre-chilled 1.5 mL microcentrifuge tubes and centrifuged at 13,000 rpm for 20 minutes at 4 $^{\circ}$ C. Supernatant was aspirated immediately and pellets stored at -80 $^{\circ}$ C until needed. On the following day, one pellet was resuspended in 1 mL cold binding buffer and protein concentration quantified using a Quick StartTM Bradford Protein Assay Kit 2 (Bio-Rad, Cat.# 500-0202). On day of assay, the following components were diluted in cold binding buffer in a 96 well 2 mL deep well polypropylene plate and incubated at room temperature on a platform rocker for 1 hour: 25 μ g membrane preparation, 1 nM [³H]ketanserin hydrochloride (PerkinElmer, Part# NET791025UC), and test compound diluted in binding buffer (DMT: 20 nM to 30 μ M; HL: 200 nM to 300 μ M; 5-HT: 100 pM to 10 μ M). Mianserin (100 μ M) was used to define nonspecific binding. Each concentration point was tested in triplicate. Samples were filtered onto a 0.25% polyethylenimine-coated UniFilter-96 GF/C microplate (PerkinElmer, Part# 6055690) using a PerkinElmer FilterMateTM cell harvester and counted in MicroScintTM-20 cocktail (PerkinElmer, Part# 6013621) on a PerkinElmer Microbeta² System at 57% efficiency. K_i values were generated using the GraphPad Prism 9.0 “one site – fit K_i ” function (GraphPad Software, Inc.). HL freebase was dissolved in DMSO and the final concentration of DMSO in assay plate was $\leq 3\%$.

Statistical analysis

Data are presented as mean $\pm$ SEM. Statistical analysis was performed using GraphPad Prism 9.0. Between group differences in variance were calculated using the nonparametric Kruskal-Wallis test. Multiple comparisons were performed using the two stage linear set-up procedure of Benjamini, Krieger, and Yekutieli. A nonparametric test was chosen for simplicity due to the nature of the data and because the assumption of homoscedasticity for ANOVA was not met. P values less than 0.05 were considered statistically significant.

RNA-seq analysis

HTSeq gene counts obtained from Novogene were used to perform tests in DESeq2 v1.29.6.⁸⁹ Prior to running tests for differential gene expression, we corrected unwanted variation (noise stemming from technical and biological factors unmodelled in the experiment) using RUVseq⁹⁰ to increase power and biological insight specific to the experimental design.⁹¹ Briefly, after normalizing the counts (upper quantile) using the RUVr function we estimated the variables that capture the unwanted variation by factor analysis on deviance residuals after a running a regression of the counts on the covariates of interest (**Sup. Fig. 19**). Those variables were

included in the design formula in DESeq2. For the tests run in DESeq2 we used un-normalized HTSeq counts after filtering genes with less than 10 reads in at least 3 samples. Statistical significance was set at $FDR < 0.1$ (Benjamini-Hochberg (BH) adjusted p value).⁹² This FDR was chosen based on a previous work in which LC-MS based proteomics and IPA analysis were used to analyze the effects of 5-methoxy-DMT on human cerebral organoids.⁵⁴ For clustering analysis (PCA and unsupervised hierarchical clustering heatmaps), we used the variance stabilized RUVseq corrected counts. Variance stabilization was performed with the functions in the vsn package.^{93,94} Hierarchical clustering was performed and plotted using the functions in the ComplexHeatmap package.⁹⁵ Briefly, we calculated the Euclidean distances and used Ward's linkage to obtain the clusters. Volcano plots were plotted with Enhanced Volcano in R.⁹⁶ Gene Set Enrichment Analysis (GSEA) was performed for the genes that passed the FDR .1 threshold in pairwise comparisons with gene ranks calculated from shrunken log fold changes⁹⁷ corrected by the corresponding FDR, in WebGestalt⁹⁸ using the Gene Ontology (GO) and KEGG pathway functional databases. Ingenuity Pathway Analysis (IPA) pathway analysis used the same data sets, but instead plugged in gene ensemble IDs, FDR, and fold change. Rnorvegicus genes were mapped to human orthologues for pathway analysis in IPA.

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DPK, KV, SB conceived of the animal experiments and were encouraged and guided by JF to perform the experiments. DPK and KV carried out the animal experiments with assistance from PE and JF. PE performed EPR for the first iteration of the experiment and DPK performed EPR for the replication. DPK performed statistics on the EPR data. DPK and KV sacrificed animals with assistance from PE and collected tissue samples. GB, DPK and CN conceived of the *in vitro* experiments and GB performed those experiments and analyzed those data. DPK extracted mRNA from the PFC and performed quality control measures (nanodrop and fragment analysis) and shipped those samples to Novogene corporation. Novogene performed total RNA sequencing. AD analyzed the sequencing data and DPK performed GO and KEGG pathway analysis and KS performed IPA pathway analysis. DPK wrote the majority manuscript and organized the figures. AD wrote the methods for sequencing. GB wrote the methods and results for the *in vitro* experiments. KV, GB, SB, CN, and AD performed critical revisions of the manuscript. All authors approved the final version of the manuscript prior to submission for publication.

Figures

Figure 1

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Figure 2

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a) Control vs PE/PSS

b) PE/PSS vs DMT

Figure 3

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a) PE/PSS vs Harmaline

b) PE/PSS vs pharmahuasca

Figure 4

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Figure 5

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K_i (nM) $\pm$ SEM

Drug	h5-HT _{2A}	r5-HT _{2A}	h5-HT _{1A}	r5-HT _{1A}
	[³ H]ketanserin	[³ H]ketanserin	[³ H]8OH-DPAT	[³ H]8OH-DPAT
5-HT	187 $\pm$ 51	614 $\pm$ 43 ⁶¹	2.67 $\pm$ 0.33 ⁶³	2.2 $\pm$ 0.5 ⁶⁴
DMT	511 $\pm$ 67	660 $\pm$ 30 ⁶²	450 $\pm$ 150 ⁶³	200 $\pm$ 10 ⁶²
Harmaline	> 10,000	7790 $\pm$ 1200 ⁶³	ND	> 10,000 ⁶²

f)

	DMT	Harmaline	5-HT
Bottom	= 0.000	= 0.000	= 0.000
Top	95.58	7.754	100.0
LogEC50	-7.702	-7.265	-9.209
EC50	1.986e-008	5.435e-008	6.187e-010
Span	= 95.58	= 7.754	= 100.0

Figure 6

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Figure 7

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Figure 8

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