Chronic, Intermittent, Microdoses of the Psychedelic *N,N*-Dimethyltryptamine Produce Positive Effects on Mood and Anxiety in Rodents

Supplementary Material
(10 pages)

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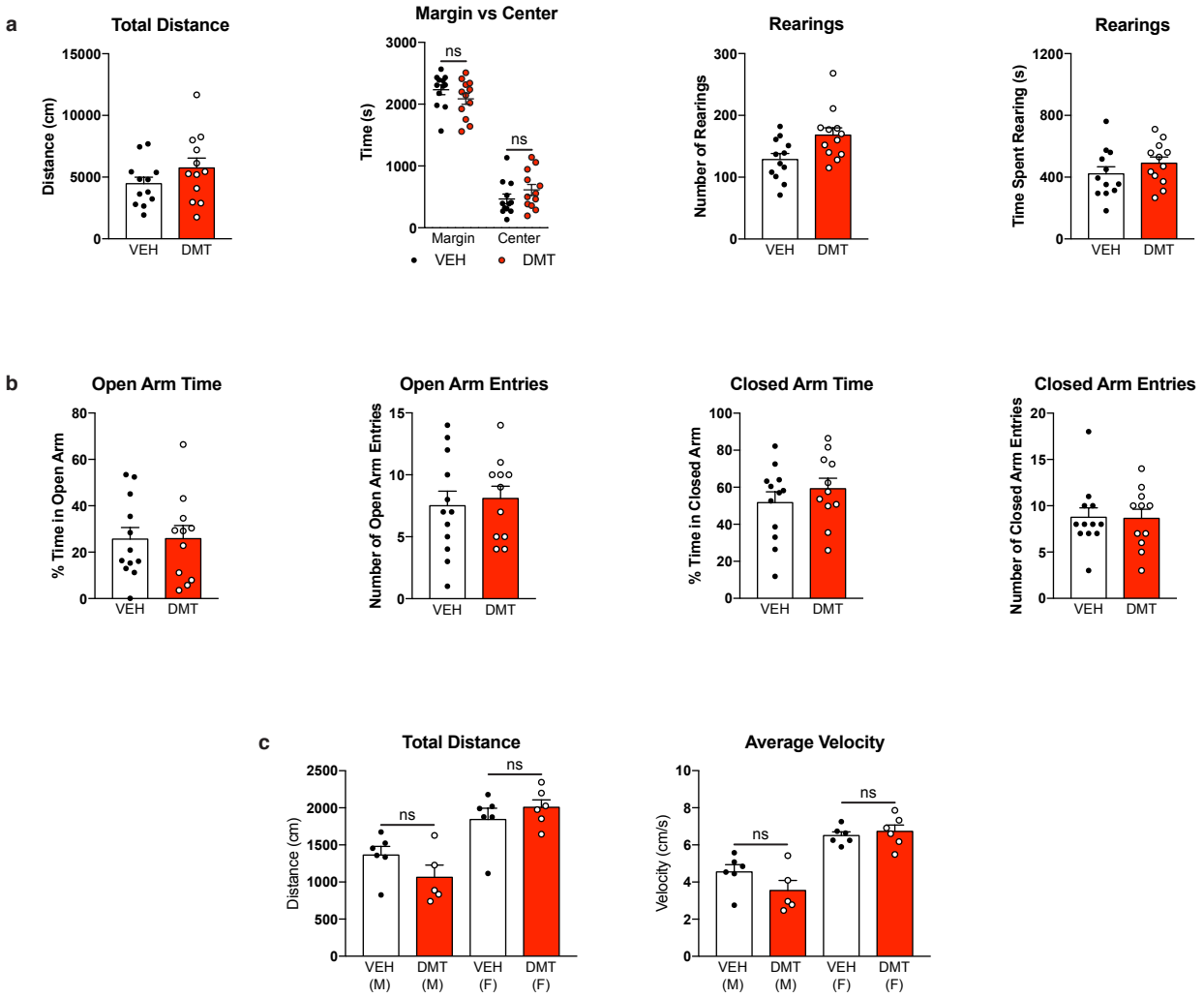
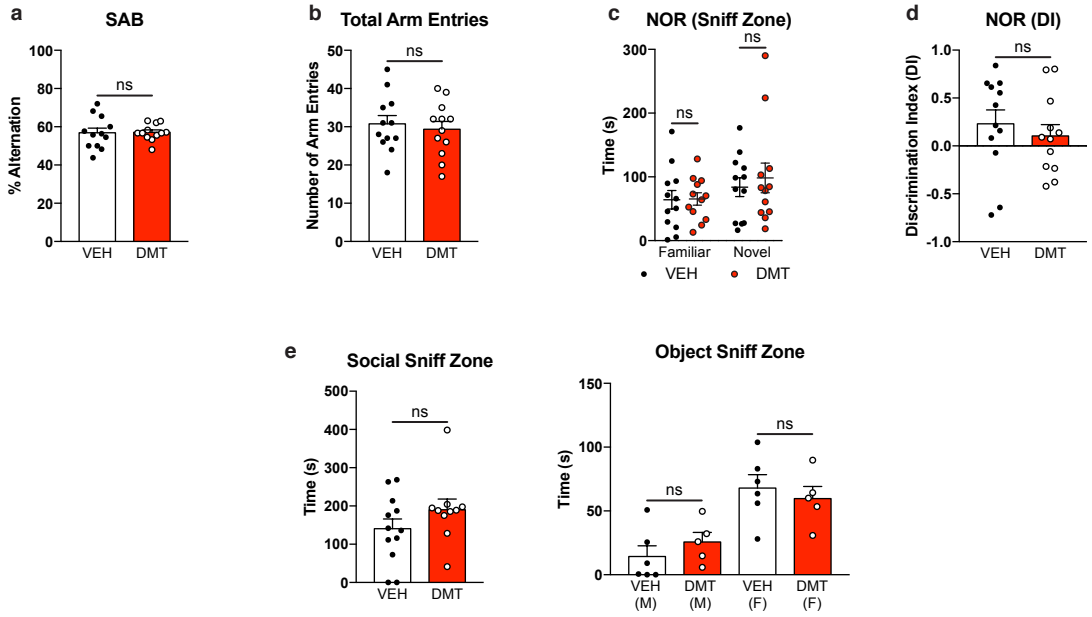
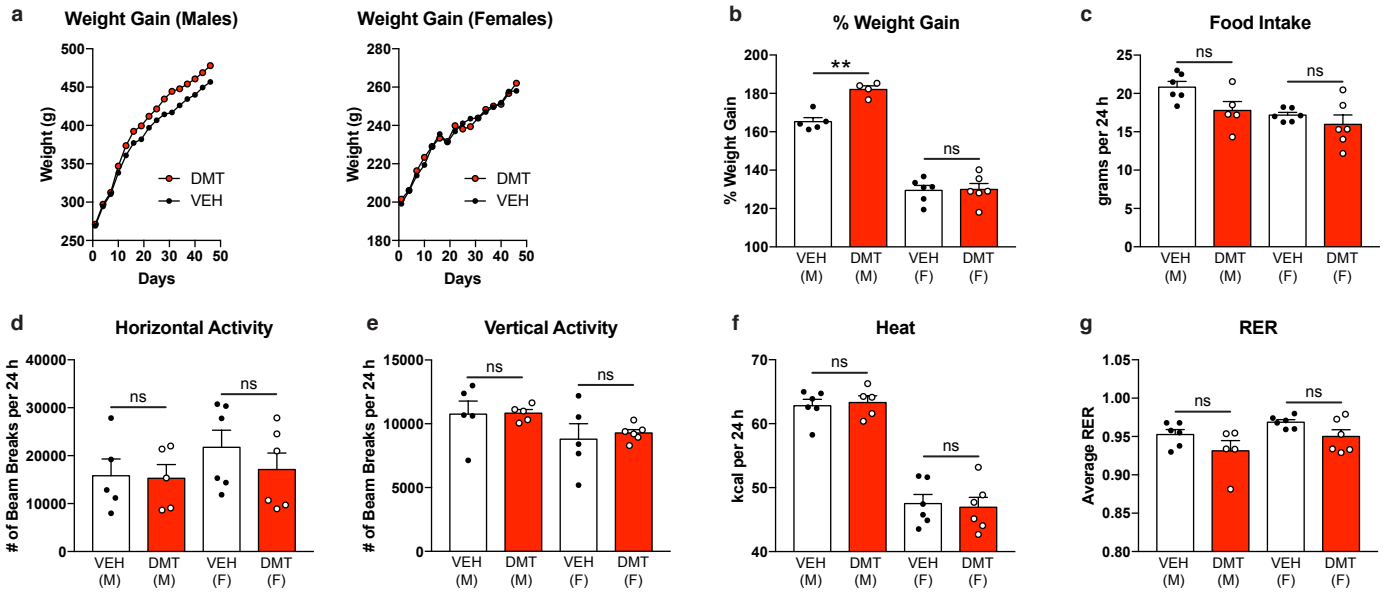


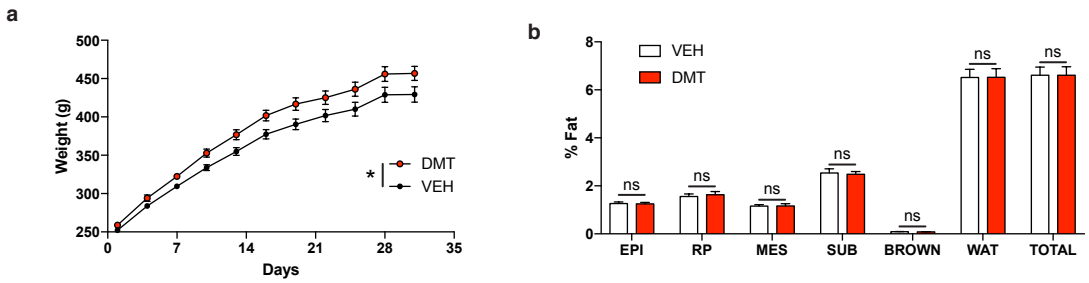
Supplementary Figure 1 | Behavioral Phenotypes measured for NIL and EPM. a, b, Results from NIL assays (a) and EPM (b) for male and female animals treated with vehicle or microdosed with DMT. Statistical analysis was not performed on these data, and instead, the results were subjected to Z-normalization and combined to produce a single NIL or EPM Score (see Statistical Methods section). **c,** Control experiments demonstrate that there is no difference in locomotor activity between the two treatment groups in the EPM. Error bars represent s.e.m., ns = not significant, M = males, F = females. See Supplementary Table 1 for details of all statistical tests.



Supplementary Figure 2 | Chronic, intermittent, low doses of DMT do not improve cognitive function or increase social interaction in rats. (**a–b**) In the spontaneous alternation behavior (SAB) paradigm, DMT- and vehicle-treated groups displayed comparable levels of percent alternation (**a**) and total arm entries (**b**). (**c–d**) DMT- and vehicle-treated groups displayed no differences in novel object recognition as determined by the total time spent in the "sniff zone" of either the novel or familiar objects (**c**) or by comparing the discrimination indices (DI) between the groups (**d**). A DI > 1, < 1, = 0 indicates a preference for the novel object, a preference for the familiar object, or no preference, respectively. (**e**) No differences were observed between treatment groups in the 3-chambered social approach with respect to the amount of time they spent in the "sniff zone" of either the conspecific or object. For SAB and NOR, n = 12 DMT-treated animals (6 male and 6 female) and n = 12 vehicle-treated animals (6 male and 6 female). For Social Interaction, n = 10 DMT-treated animals (5 male and 5 female) and n = 12 vehicle-treated animals (6 male and 6 female); Error bars represent s.e.m., ns = not significant. See Supplementary Table 1 for details of all statistical tests.



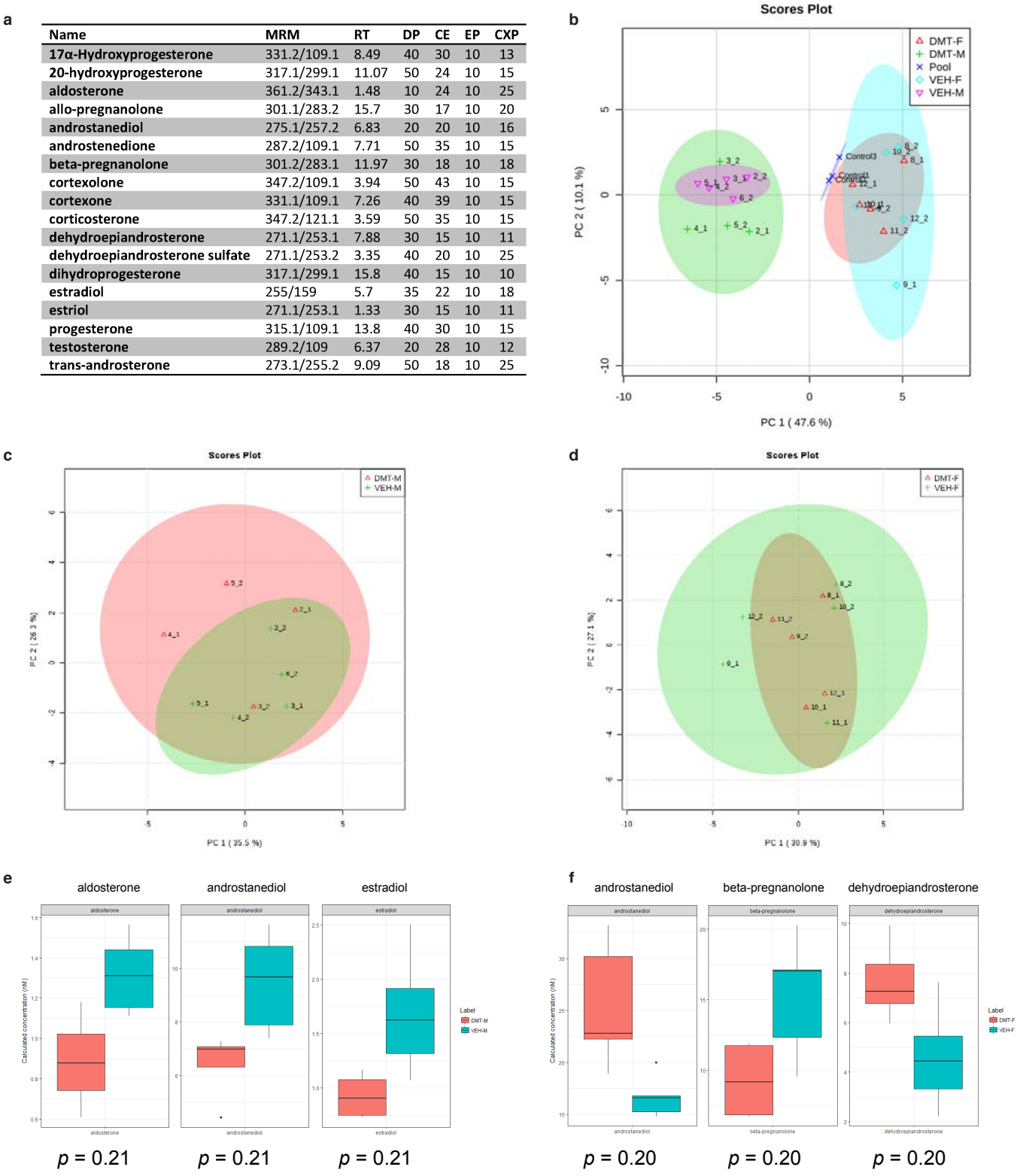
Supplementary Figure 3 | Chronic, intermittent, low doses of DMT produce sexually dimorphic changes in metabolism. (**a**) DMT administered at 1 mg/kg every third day causes accelerated weight gain in male, but not female rats. Error bars removed for clarity. (**b**) Percent weight gain over the course of a 7-week microdosing experiment. (**c**) Average food intake over the course of 48 h following 7 weeks of DMT microdosing was not affected by DMT treatment. (**d–f**) Microdosing did not impact horizontal activity (**d**), vertical activity (**e**), dissipation of heat (**f**), or respiratory exchange rate (RER) (**g**). Error bars represent s.e.m., ns = not significant, $^{**}p < 0.01$. See Supplementary Table 1 for details of all statistical tests.



Supplementary Figure 4 | Chronic, intermittent, low doses of DMT increase weight in male rats, but not adiposity. a, Male rats were weighed and treated with 1 mg/kg DMT or vehicle every third day for 4 weeks. A significant difference was observed between the two groups ($p = 0.026$, two-way repeated measures ANOVA). **b,** At the conclusion of 4 weeks of dosing, the animals were sacrificed and their fat pads dissected and weighed. Data are presented for each fat pad (or combination of fat pads) as a percentage of the total body weight. No significant differences were observed between treatment groups. EPI = epididymal; RP = retroperitoneal; MES = mesenteric; SUB = subcutaneous; BROWN = brown fat; WAT = sum of white adipose tissue (EPI + RP + MES + SUB); TOTAL = sum of all fat pads. n = 12 for each treatment group. Error bars represent s.e.m., ns = not significant, $^*p < 0.05$. See Supplementary Table 1 for details of all statistical tests.

a

Name	MRM	RT	DP	CE	EP	CXP
17α-Hydroxyprogesterone	331.2/109.1	8.49	40	30	10	13
20-hydroxyprogesterone	317.1/299.1	11.07	50	24	10	15
aldosterone	361.2/343.1	1.48	10	24	10	25
allo-pregnanolone	301.1/283.2	15.7	30	17	10	20
androstanediol	275.1/257.2	6.83	20	20	10	16
androstenedione	287.2/109.1	7.71	50	35	10	15
beta-pregnanolone	301.2/283.1	11.97	30	18	10	18
cortexolone	347.2/109.1	3.94	50	43	10	15
cortexone	331.1/109.1	7.26	40	39	10	15
corticosterone	347.2/121.1	3.59	50	35	10	15
dehydroepiandrosterone	271.1/253.1	7.88	30	15	10	11
dehydroepiandrosterone sulfate	271.1/253.2	3.35	40	20	10	25
dihydroprogesterone	317.1/299.1	15.8	40	15	10	10
estradiol	255/159	5.7	35	22	10	18
estriol	271.1/253.1	1.33	30	15	10	11
progesterone	315.1/109.1	13.8	40	30	10	15
testosterone	289.2/109	6.37	20	28	10	12
trans-androsterone	273.1/255.2	9.09	50	18	10	25

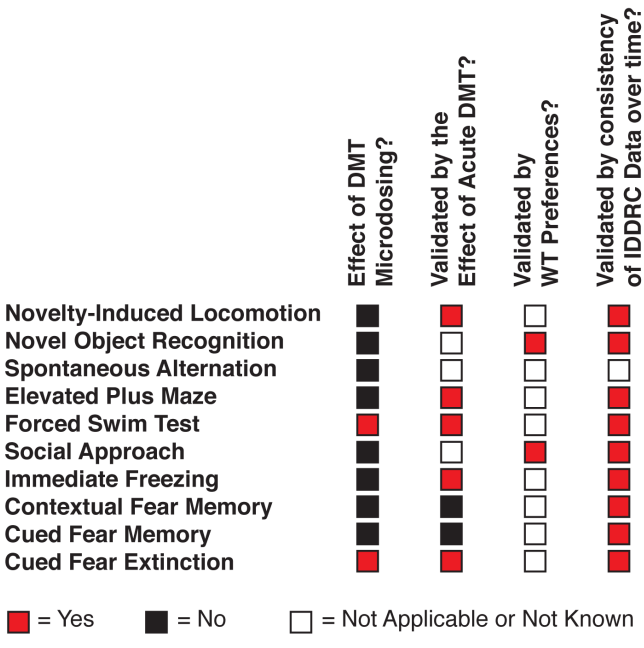


Supplementary Figure 5 | Chronic, intermittent, low doses of DMT produce minimal changes in serum steroid levels. a, We attempted to quantify 30 steroids in the serum of DMT-microdosed rats, however, 12 were below the limit of detection. The 18 steroid analytes detected, MRM transitions, retention times (RT), declustering potentials (DP), collision energies (CE), entrance potentials (EP), and cell exit potentials (CEP) are listed. **b,** Principal components analysis revealed large differences between male and female samples. Data collected for pooled samples at the beginning, middle, and end of the mass spectrometry experiment were consistent with each other and demonstrate minimal assay drift. **c–d,** Principal components analysis for male (**c**) and female (**d**) samples show that DMT-treatment produced minimal changes in serum steroid profiles. Principal components analyses were $\log_{10}$ transformed and pareto scaled. **e–f,** Levels of three steroids in male (**c**) and female (**d**) samples following DMT microdosing are shown. For box plots: horizontal line = median, 50% quatile; lower hinge = 25% quartile; upper hinge = 75% quartile; lower whisker = smallest observation greater than or equal to lower hinge – 1.5 x IQR; upper whisker = largest observation less than or equal to upper hinge + 1.5 x IQR; Dots = outliers defined by being either greater than Q3 – 1.5 x IQR or less than Q1 – 1.5 x IQR. Q3 = third quartile; Q1 = first quartile; IQR = interquartile range = Q3 – Q1. See Supplementary Table 1 for details of all statistical tests.

SUPPLEMENTARY METHODS

Behavior Methods (General). When designing this study, we realized that the chronic nature of the microdosing treatment schedule would likely result in large cohort-to-cohort variation due to uncontrollable changes in experimental conditions such as timing of behavioral tests, changes in vivarium personnel, construction, etc. Therefore, we opted to perform a well-powered single-cohort study. Based on the amount of time required to perform the planned behavioral experiments, the maximum number of animals that we could test in a single cohort was 24. Next, we performed a power analysis using pilot data and our previous studies involving acute administration of DMT to estimate effect sizes for the various behavioral tests. We concluded that 6 animals per group would be sufficient to detect statistical differences between treatment groups in most paradigms, with the exception being that 12 animals would likely be needed for fear extinction. Because it is well known that males and females often exhibit differential responses to antidepressant and anxiolytic treatments, we opted to conduct a well-powered single-cohort study capable of detecting sexually dimorphic phenotypes (e.g., N = 6 per sex/treatment group x 4 groups = 24 animals) rather than a single-sex dose-response study (e.g., N = 6 males (or females) x 4 treatments (VEH, high, med, and low dose of DMT) = 24 animals). We reasoned that if the males and females of each treatment group responded similarly, the data could be combined to increase statistical power and reduce the chance of making a Type II error.

Due to the limited number of animals that we were capable of testing in a single cohort, we could not include separate control compounds for various behaviors. However, we were assured that the behavioral assays were working properly in our hands for several reasons. First, we used equipment, analysis, and behavioral protocols that have been well-validated by the UC Davis Intellectual and Developmental Disabilities Research Center (IDDRC) Rat Behavioral Testing Core. The use of a core facility improves rigor and reproducibility by ensuring standardization of housing conditions, equipment, lighting, measurements, data analysis, etc. so that the consistency of data from control groups is maintained over time.[1] Second, we confirmed that the VEH-treated group exhibited typical, expected values for all behavioral tests and that expected wild type (WT) preferences were observed (e.g., typical total amount of locomotion in NIL, typical % alternation in SAB, typical % open arm time in EPM, reasonable discrimination index in NOR, social preference exhibited in 3-chambered social approach, typical % freezing in fear conditioning, etc.). Finally, these behavioral studies were conducted at approximately the same time, by the same experimenter (CJB), using the same equipment, as our previously reported study on the acute effects of DMT in rats.[2] In that previous study, DMT caused robust anxiogenic-like effects in the NIL and EPM paradigms, while also increasing immediate freezing following footshocks. The accompanying graphic depicts how each behavioral test used in this study was validated. Spontaneous alternation behavior was the only test not specifically validated by at least one of the three methods outlined in the graphic. However, the percent alternation observed was typical of similar experiments reported in the literature. The combination of validation methods used here supports the conclusion that the lack of behavioral responses in several paradigms following DMT microdosing are not likely due to Type II errors.



Novelty-Induced Locomotion (NIL). Rats were allowed to acclimate to the test room for 10 min prior to being gently placed into the center of an AccuScan Instruments (Columbus, OH) open field chamber (Digiscan Animal Activity Monitor model #RXYZCM(16)CCD) and allowed to freely explore the chamber for 45 min. At the conclusion of the test, animals were returned to their home cages and the test chambers cleaned with 10% Nolvasan. Horizontal motion and rearings were recorded in 1 min intervals for the duration of the test and analyzed using the program Integra. The margin of the arena was defined as being 10 cm from the wall. The open field chamber measured 41.9 cm L × 41.9 cm W × 28.6 cm H and was illuminated to between 25 and 30

lx. The raw data for several phenotypes (i.e., total distance traveled, time spent in the center of the arena, number or rearings, and time spent rearing) are presented in Supplementary Figure 1a, but statistics were not performed. Instead, each of these related behavioral measurements were subjected to Z-score normalization and an overall NIL Score was calculated (See Statistical Analysis Section). Anxiolytic-like behaviors (e.g., increased locomotion, increased time spent in the center of the arena, increased number of rearings, and increased time spent rearing) were arbitrarily defined as positive Z-scores.

Novel Object Recognition (NOR). The NOR chamber measured 53.3 cm L x 53.3 cm W 34.3 cm H, was illuminated to between 25 and 30 lx, and contained a pair of identical objects (either a 4 inch cone or a 3–4 inch jar filled with cement). On the first day, rats were allowed to freely explore the objects for 10 min before being returned to their home cages. After 24 h, rats were reintroduced to the NOR chamber, but one of the objects was replaced with a new, distinctly different object. EthoVision XT (version 9) software was used to assess the subject rat's preference for the novel vs the familiar object by quantifying the amount of time the nose point spent within the "sniff zone"—defined as a circle surrounding the object with a radius of 2 cm. Additionally, a discrimination index (DI) was calculated as (Time Exploring$^{\text{Novel Object}}$ – Time Exploring$^{\text{Familiar Object}}$) / Total Time Exploring$^{\text{Both Objects}}$. The entire apparatus was cleaned with 10% Nolvasan between trials.

Spontaneous Alternation Behavior (SAB). Rats were placed on one arm of a T-maze facing the central node and allowed to freely explore for 10 min. Animal movement was recorded and analyzed by an experimenter blinded to treatment after the completion of the behavioral task. Percent alternation was calculated by dividing the number of times the rodent sequentially accessed the 3 distinct arms of the maze by the total number of arm entries minus one. The entire apparatus was cleaned with 10% Nolvasan between trials.

Elevated Plus Maze (EPM). The EPM apparatus consisted of a black plus-shaped plastic platform positioned 50 cm above the ground and illuminated to between 20 and 25 lux. Two opposite arms of the maze possessed vertical walls measuring 31.75 cm high, with the other two arms being open with unprotected edges. Rats were placed in the center of the maze facing an open arm and allowed to explore freely for 5 min. At the conclusion of the test, rats were returned to their home cages and the apparatus was cleaned with 10% Nolvasan. Animal movement was recorded and analyzed during the trial using EthoVision XT (version 9) software. The raw data for several phenotypes (i.e., percentage of open arm time, number of open arm entries, percentage of closed arm time, and number of closed arm entries) are presented in Supplementary Figure 1b, but statistics were not performed. Instead, each of these related behavioral measurements were subjected to Z-score normalization and an overall EPM Score was calculated (See Statistical Analysis Section). Anxiolytic-like behaviors (e.g., increased time spent in the open arms, increased number of open arm entries, decreased time spent in the closed arms, and decreased number of closed arm entries) were arbitrarily defined as positive Z-scores.

Forced Swim Test (FST). The modified FST was performed as reported previously.[3] The FST apparatus consisted of a clear Plexiglas cylinder measuring 40 cm tall, 20 cm in diameter and filled with 30 cm of 24 ± 1ºC water. Fresh water was used for every rat. Animals were subjected to a pre-test phase in which they were placed in the cylinder for 15 mins before being dried and returned to their home cage. Twenty-four hours later, rats were again placed in the FST apparatus for 5 mins and their activity was video recorded. Each video was scored for immobility, swimming, and climbing behavior by two trained observers blinded to treatment condition (Pearson correlation coefficients = 0.7, 0.7, 0.9, respectively), and these results were averaged. The dominant behavior of the animal (i.e., immobility, swimming, or climbing) was determined every 5 sec and quantified as a "count." As the experiment lasted for 5 mins, the sum of the counts for all four behaviors equals 60.

3-Chambered Social Approach. The social approach apparatus consisted of three identical chambers separated by removable gates, with each chamber measuring 101.6 cm L x 33.3 cm W 33.7 cm H. Each chamber was illuminated to between 25-30 lux and housed a cage for holding "social animals" or objects. The cages restrained the movement of the social animals while still enabling interaction with the test animals. One day prior to the experiment, social animals were acclimated to the cages of the social approach chamber in two 15-min sessions. On the test day, subject rats were habituated to the apparatus with no social animal or object present for 10 mins, before being gently corralled back into the center chamber and the gates closed. A novel

object was then placed in one of the adjacent chambers, and a social animal placed in the other chamber. The gates were then opened and the subject rat was allowed to freely interact with the social animal or object, and their activity was video recorded. EthoVision XT (version 9) software was used to assess the subject rat's preference for social interaction. Time spent in the "sniff zone" was quantified using the nose point of the animal. The sniff zone was defined as 2 cm from the edge of the cage holding either the social animal or the object. Social animals were the same sex and approximate age as subject rats. The entire apparatus was cleaned with 10% Nolvasan between trials.

Fear Conditioning. Rats were placed in a fear conditioning apparatus (Med Associates model # MED-VFC2-SCT-R) for 3.5 min prior to three presentations of auditory cues (80 dB white noise, 30 s), each coterminating with a foot shock (0.8 mA, 2 s.) and spaced 90 s apart. The fear conditioning apparatus consisted of a 30.5 cm × 24.1 cm × 21 cm internal soundproof chamber, with metal grated floors, an infrared camera, a sound generator, and a light source. After the last shock, the animals remained in the chambers for an additional 2 min before being returned to their home cages. During fear conditioning, the apparatus was illuminated to 100 lx and did not contain any additional odor cues. The apparatus was cleaned with 70% EtOH in between trials. All fear conditioning and extinction experiments were performed between the hours of 08:00−11:00. Freezing behavior was scored using Med Associates Video Freeze software v2.25 (motion threshold = 18 au, detection method = linear, minimum freeze duration = 30 frames, which is equal to a 1 s freeze). Pre-training was defined as the 3.5 min prior to receiving footshocks, while post-training refers to the 2 min that the animal remained in the fear conditioning chamber after the last foot shock.

Contextual Fear Memory. On the first day after fear conditioning, contextual fear memory was assessed by exposing the animals to the conditioning context for 10 min and measuring their freezing behavior over this time. The apparatus was cleaned with 70% EtOH in between trials.

Cued Fear Memory and Extinction Training. On the second day after fear conditioning, the animals were administered DMT and then placed back in their home cages. On the third day after fear conditioning, cued fear memory was assessed by exposing the animals to a novel context (lights off, A-frame insert, smooth plastic floor insert, additional vanilla odor) for 2 min prior to 8 presentations of auditory cues (80 dB white noise, 30 s) spaced 30 s apart. Freezing responses for cue testing are presented as the percentage of time spent freezing during the last 4 auditory presentations. Cued fear memory testing also served as cued fear extinction training. The apparatus was cleaned with 70% EtOH in between trials.

Cued Fear Extinction Memory. On the fourth day after fear conditioning, the cued fear memory procedure was repeated.

Comprehensive Laboratory Animal Monitoring System (CLAMS). Rats were singly housed in CLAMS chambers (Columbus Instruments) for ~48 h and fed powdered chow diet (2018 Teklad Global 18% Protein Rodent Diet) *ad libitum*. Cage sensors monitored food consumption as well as horizontal and vertical activity. Oxygen consumption and carbon dioxide production were measured and used to calculate energy expenditure (i.e., heat dissipated) and respiratory exchange ratio (RER = VCO_2/VO_2).

Tissue Collection. After completion of the behavioral and metabolic experiments, the animals were sacrificed via decapitation. Blood and brain tissue were collected for metabolomics, Golgi-Cox staining, and gene expression studies as described below.

Golgi-Cox Staining. Brain tissue was prepared following the protocol outlined in the FD Neurotechnologies Rapid GolgiStain Kit (FD Neurotechnologies) with slight modifications. Brains were stored in solution C for 2 weeks prior to slicing into 130 µm sections using a vibratome. These slices were placed onto microscope slides that were pre-coated with (3-aminopropyl)triethoxysilane. Slices were air dried for a week before staining. Slides were immersed in water twice for 2 minutes, DE solution for 10 minutes, and then water for 2 minutes. After this, slides were immersed sequentially in 25% ethanol for 1 minute, 50% ethanol for 4 minutes, 75% ethanol for 4 minutes, 95% ethanol for 4 minutes, and 100% ethanol for 4 minutes. Slides were then briefly dipped into xylenes before being mounted using DPX Mountant For Histology (Sigma), air-dried, and imaged on a Zeiss AxioScope. Spines were traced using Neurolucida software (version 10) at 100x magnification. Data acquisition and analysis was performed by an experimenter blinded to treatment

conditions. Data represent individual neurons taken from 2 different animals per treatment. Representative images were acquired with a 60x oil immersion objective (1.42 NA) using the 488 nm laser and transmission detector on an Olympus FV1000 confocal microscope.

ddPCR. Tissue from the PFC of rats subjected to chronic, intermittent, low doses of DMT or vehicle was removed and lysed using QIAzol Lysis Reagent (QIAGEN). Extraction of RNA was accomplished using the RNeasy isolation kit (QIAGEN) following the instructions of the manufacturer. The resulting RNA was converted to cDNA using the iScript cDNA Synthesis Kit (BioRad). The cDNA was diluted 1:100 prior to droplet digital PCR (ddPCR). Droplets containing cDNA, ddPCR master mix (BioRad), and TaqMan probes (ThermoFisher) were generated using the QX200 Droplet Digital PCR System (BioRad). Following PCR amplification, the signal of the gene of interest (FAM-labeled) was quantified and normalized to the housekeeping gene ESD (VIC-labeled). Taqman probes included those for FOS (ThermoFisher, Rn02396759_m1), EGR1 (ThermoFisher, Rn00561138_m1), EGR2 (ThermoFisher, Rn00586224_m1), ARC (ThermoFisher, Rn00571208_g1), BDNF (ThermoFisher, Rn02531967_s1), HTR2A (ThermoFisher, Rn00568473_m1), and ESD (ThermoFisher, Rn01468295_g1).

Metabolomics. Blood was allowed to clot for 15 min at room temperature, centrifuged at 1,000 x g for 10 min at 0ºC, and the resulting serum was transferred to a new tube and stored at -80ºC until analysis via liquid chromatography-mass spectrometry. Samples were extracted by the *Shake and Shoot* protocol[4] with minor modification. Briefly, samples were allowed to thaw on wet ice and then inverted five times to homogenize the serum. To a 1 mL 96-well plate (Eppendorf, Hamburg, Ger.) was added 50 μL of sample serum followed sequentially by 25 μL anti-oxidant solution, 25 μL of surrogate standards in methanol, 25 μL of CUDA and PHAU standards in methanol, and 125 μL acetonitrile/methanol (1:1). The plate was then vortexed for 30 s and centrifuged for 5 min to pellet precipitated proteins in a Genevac EZ-2 centrifugal evaporator (Ipswich, UK). Supernatant was filtered through an Agilent PVDC 0.2 μm filter-plate (Santa Clara, CA). The plate was then sealed and placed in the autosampler and maintained at 6ºC. Extracted samples were analyzed for steroids by liquid chromatography-mass spectrometry (LC-MS/MS). A Waters ACQUITY i-Class LC system (Milford, MA) was coupled to a Sciex 6500+ QTRAP (Redwood City, CA) operated in multiple reaction monitoring (MRM) mode. An Agilent InfinityLab Poroshell 120 EC-C18 (2.1 x 100 mm, 1.9 μm) column was used with an acetonitrile/water gradient. Formic acid (0.1% v/v) was added to both mobile phases. A full list of steroid analytes, MRM transitions, retention times, declustering potentials, collision energies, entrance potentials, and cell exit potentials can be found in Supplementary Figure 5a.

Fat Pad Analysis. Male rats were treated with DMT (12 rats) or vehicle (12 rats) every third day for 4 weeks, and weights were recorded at regular intervals. At the completion of the experiment, the animals were sacrificed via decapitation and their fat pads dissected and weighed. Fat pad weights are presented as a percentage of total body weight.

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