SUPPLEMENTARY INFORMATION

The Impacts of Adolescent Cannabinoid Exposure on Striatal Anxiety and Depressive-Like Pathophysiology Are Prevented by the Antioxidant *N*-Acetylcysteine

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Supplemental Materials

Animals and housing: Male Sprague-Dawley rats were pair-housed in controlled environmental conditions (12 h light/dark cycle) with free access to food and water. All procedures and protocols were approved by appropriate Governmental and Institutional guidelines.

Drugs: THC (Cayman Chemical) was dissolved in ethanol, cremophor, and saline (1:1:18). Ethanol was evaporated using nitrogen gas to remove it from the final THC solution. NAC (Millipore Sigma) was diluted in tap water.

Adolescent treatment protocol: Rats were treated twice daily from postnatal day (PND) 35 to 45 with escalating doses of THC (2.5 mg/kg; Days 35-37; 5 mg/kg; Days 38-41; 10 mg/kg, Days 42-45; i.p.) or vehicle. Between PND 35 and 65, rats had *ad libitum* access to NAC solution (0.9 g/L) or vehicle. The NAC solution was freshly prepared every other day.

The adolescent THC exposure protocol was chosen based on the previously published literature, reporting that such dosing regimen alters the physiological brain development and induces long-lasting pathological phenotypes (1–3). The increasing dosing schedule was used to overcome the potential CB1 receptor desensitization and tolerance to THC. This THC protocol is designed to mimic heavy cannabis consumption in youth. The NAC exposure protocol was adapted from previous studies (4–8). NAC was administered in drinking water to minimize any potential stress factors. The dose of 0.9 g/L was chosen based on the previously published literature, which reported such concentration to be safe and effective in the early stages of life. NAC administration started in adolescence (PND 35) and continued until young adulthood (PND 65). This protocol was designed to counteract the abnormalities induced by THC during the 11-day exposure period and the following maladaptive processes resulting from the disrupted brain development. The behavioral assays, electrophysiological recordings, and molecular investigations (i.e., MALDI-

IMS and Western Blot experiments) started after a drug-free washout period (PND 75). An experimental timeline is represented in Supplemental Fig.1.

Behavioural tasks:

Elevated Plus Maze (EPM): EPM is a validated test to assess anxiety in rodents. The apparatus (black acrylic) consisted of a plus shaped elevated above the floor by 50 cm, with 4 arms (10x50 cm) and a center platform. Two opposite arms were enclosed with 40 cm high walls while other two arms were opened (except for 1 cm high ledge). A rat was placed on a closed arm, and was allowed to freely explore the maze for 10 min. The apparatus was cleaned between sessions with a 70% ethanol solution to avoid olfactory cue bias. The number of entries and the time spent in closed and open arms were video-recorded and analyzed offline using Behaview software.

Novelty Suppressed Feeding (NSF): NSF task is a depression-related test in which rodents face a conflict between a novel environment and feeding following prolonged food deprivation. Subjects were food-deprived for 24 hours, with free access to water. On the test day, a rat was placed in the corner of a black acrylic box (80x80 cm) filled with a layer of wood bedding, facing some food pellets in a dish at the center of the arena. The testing room was dark, and the arena was brightly illuminated with 700/800 lumens at the floor level. Rats freely explored the apparatus for 10 minutes, and their behavior was video-recorded and analyzed offline using Behaview software. The latency to eat, defined as the total time it takes for the animal to enter the center of the arena and bite the food pellet, the latency to approach the food from the start of the test, and the total grams of food consumed over 10 minutes were examined. The wood bedding was changed, and the apparatus was cleaned between sessions with a 70% ethanol solution to avoid olfactory cue bias.

Porsolt Forced Swimming Test (FST): FST is a test to assess learned helplessness based on passive immobility as an index of behavioral despair. Rats were placed in a cylinder (70x19 cm) filled with water (24.5±0.5 °C) for 15 minutes. Immobility, defined as the complete absence of movements except those required to float, was video-recorded and analyzed offline using Behaview software.

Contextual Fear Memory Conditioning (CFC): CFC is a conditioning paradigm during which rats learn to associate a context with an aversive stimulus. The apparatus consisted of two distinct conditioning chambers (40x40x50 cm) with distinctive visual cues on three walls and a Plexiglass transparent front wall. Context A comprised black dots on a white background, while context B comprised black and white stripes. Rats were randomly assigned to the context, and the designated environment had a metallic grid floor. The apparatus was cleaned with a 70% ethanol solution between sessions to avoid olfactory cue bias. In the conditioning phase, 10 subthreshold foot shocks (0.4 mA, 1 sec) were randomly delivered over 30 minutes. The following day, on the recall phase, rats were exposed to the previously assigned context for 10 minutes. Freezing behavior, as a complete absence of movements except respiration for at least 1 second, and rearing behavior were video-recorded and analysed offline using Behaview software.

Matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS): MALDI-IMS relative quantification of biomarkers associated with cannabis-related dysregulations in AcbSh and AcbC was carried out as previously described (9,10). Rats received an overdose of sodium pentobarbital (240 mg/kg, i.p., EuthanylTM), and brains were removed and flash frozen. Two matrices, ZnO-NP (Sigma-Aldrich, St. Louis, MO) or FMP-10 (TAG-ON, Uppsala, Sweden), were used to quantify selected biomarkers. Brains from each experimental group were sectioned with a cryostat (CM 1850, Leica Biosystems, Wetzlar, Germany) to a

thickness of 10 µm (for ZnO-NP experiments) or 12 µm (for FMP-10 experiments) and mounted onto the indium-tin-oxide (ITO) coated glass slides (Hudson Surface Technology, Old Tappan, NJ). Tissue sections containing AcbSh and AcbC were obtained at AP: +1.6 to +2.5 mm from bregma, according to the Atlas of Paxinos and Watson (11). Prior to matrix deposition, slides were desiccated at room temperature. ZnO-NP (1.0 mg/ml in 50% ACN) or FMP-10 (1.8 mg/ml in 70% ACN) dispersion was sprayed onto tissue sections using a TM-Sprayer (HTX Technologies, Chapel Hill, NC). ZnO-NP was sprayed at the following conditions: flow rate at 0.05 ml/min; nozzle temperature at 65°C, moving nozzle velocity of 1200 mm/min, 32 passes, 3 mm line spacing and pressure of 10 psi. FMO-10 was sprayed at the following conditions: flow rate at 0.08 ml/min; nozzle temperature at 82°C, moving nozzle velocity of 1100 mm/min, 30 passes, 2 mm line spacing and pressure of 6 psi. For MS mass calibration, 0.3 µL of 6.0 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA, Sigma-Aldrich, St. Louis, MO) solution containing ACN/H_2O/TFA (50/50/0.1%, vol) was spotted on each slide, near the tissue sections. A Sciex 5800 MALDI TOF/TOF mass spectrometer (Framingham, MA) equipped with a 349 nm Nd: YLF laser with a pulse rate at 400 Hz was used and images were acquired with Sciex TOF-TOF Series Explorer and TOF-TOF Imaging. Mass spectra were acquired from a *m/z* range of 50 to 500 Da (ZnO-NP) and a *m/z* range of 300-1000 Da (FMP-10) in the positive mode, and the spatial resolution for IMS was 70 µm. For each pixel, the mass spectra were acquired by averaging signals at 400 shots. The laser intensity for IMS was optimized based on a balance between peak resolution and signal-to-noise ratio. MSiReader (1.02, North Carolina State University) was used to visualize ion distributions within the tissue sections and to export the data from AcbSh and AcbC for quantitation. Analyses were performed using a custom-made script in Matlab: https://github.com/MHSarikahya/MALDI-IMS-Script. Biomarker investigations were based on

the previously published literature related to the THC pathology and/or oxidative stress. Given that adolescent THC exposure has been found to alter multiple neurotransmitters signaling (e.g., GABA, dopamine, glutamate, serotonin) (1,2,12), neurotransmitters and their precursors or metabolites were examined. Additional related molecules or aminoacids were also included. Specifically, the following biomarkers were quantified: Glutamate (*m/z*=146.95), Arginine $[M+K]^+$ (*m/z*=213.30), N-acetylaspartate $[M+Na]^+$ (*m/z*=198.13), GABA (*m/z*=353.16), Homovanillic acid (*m/z*=450.17), Aspartic acid (*m/z*=132.99), Dopamine (*m/z*=674.25), DOPAL (*m/z*=673.22), DOPAC (*m/z*=689.26), Glycine (*m/z*=343.14), Creatine (*m/z*=399.18), Creatinine (*m/z*=381.19), Alanine (*m/z*=357.2), Tyramine (*m/z*=405.19), Taurine (*m/z*=393.17), Norepinephrine (*m/z*=690.25), Serotonin (*m/z*=711.23), Spermine (*m/z* 201.92). The areas of the peaks were integrated, and the peak area ratios between each treatment (THC, NAC, THC/NAC) and vehicle were calculated for subsequent statistical analysis.

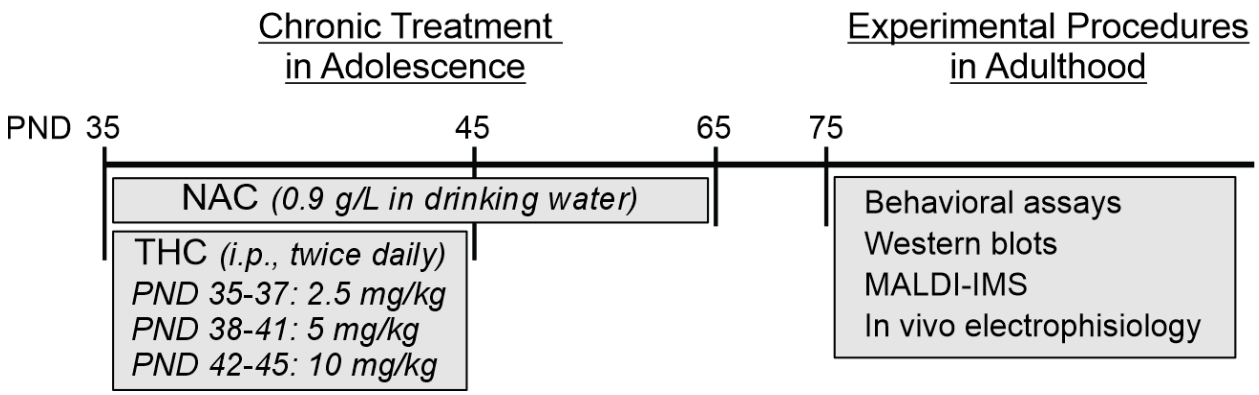
Western blots: Rats received an overdose of sodium pentobarbital (240 mg/kg, i.p., EuthanylTM) and brains were removed and flash frozen. Bilateral micro-punches of AcbSh and AcbC (AP: +1.6 to +2.5 mm from bregma) were obtained and homogenized for protein isolation in Nonidet P-40/SDS lysis buffer (150 mM NaCl, 20 mM Tris pH 7.4, 1% Nonidet P-40, 10% glycerol, and 0.1% SDS) containing protease and phosphatase inhibitors (Halt 100 × inhibitor mixture, ThermoFisher). The western blotting procedure was performed by loading 20 µg of protein per well in 6%, 8%, 10%, or 15% acrylamide SDS-PAGE gels. The gel percentage was determined based on the molecular weight of the protein examined. Samples were subjected to electrophoresis in a Bio-Rad Mini Protein 3 Western blotting apparatus with Tris/glycine/SDS buffer (Bio-Rad Cube Solutions) at 125V for 1.5h. The protein transference into nitrocellulose membranes was performed using the Trans-Blot Turbo Transfer System (Bio-Rad) at 2.5 A for 15-20 min.

Membranes were blocked for 1 hour at room temperature with 2.5% or 5% non-fat dry milk in Tris buffered Saline with Tween 20 (TBS-T). Then, membranes were incubated overnight at 4°C in a solution of TBS-T containing a primary antibody of interest. Primary antibody host species, dilutions and sources were as follows: α-tubulin (mouse, 1:500, Santa Cruz Biotechnology; rabbit, 1:2000, Proteintech), p-mTOR (rabbit, 1:1000, Cell Signaling), t-mTOR (rabbit, 1:1000, Cell Signaling), p-Akt-Thr308 (rabbit, 1:750, Cell Signaling), t-Akt (mouse, 1:1000, Cell Signaling), D1R (rabbit, 1:750, Millipore Sigma), D2R (mouse, 1:500, Millipore Sigma), GAD65 (rabbit, 1:1000, Cell Signaling), BDNF (rabbit, 1:500, Abcam), mGluR2/3 (rabbit, 1:500, Millipore Sigma), p-ERK1/2 (rabbit, 1:1000, Cell Signaling), t-ERK1/2 (rabbit, 1:1000, Cell Signaling). On the following day, membranes were washed and incubated for 2 hours at room temperature in a solution of 2.5% BSA or 5% non-fat dry milk in TBS-T containing secondary antibodies. Species-appropriate fluorophore-conjugated secondary antibodies (LI-COR IRDye 680RD and IRDye 800CW; Thermo Scientific) were used at the dilutions of 1:5000 or 1:3000, respectively. Membranes were scanned using a LI-COR Odyssey Infrared Imaging System and densitometry measurements were obtained using Image Studio analysis software. Each sample's target protein band was normalized to the intensity of the respective α-tubulin band.

In vivo electrophysiology: *In vivo* single-unit electrophysiological recordings of putative GABAergic neurons in AcbSh and AcbC were performed in urethane anesthetized (1.4 g/kg, i.p.) rats. Rats were placed in a stereotaxic apparatus (KOPF instruments) with body temperature maintained at 37±1ºC by a heating pad. The scalp was retracted, and one burr hole was drilled above the targeted area (Acb: AP: +1.6 to +2.5 mm, L: 0.8 to 1.5 mm from bregma), according to the Atlas of Paxinos and Watson (11). Extracellular single-unit activity of putative GABAergic neurons in AcbC (DV: 5.5 to 7 mm from the dural surface) and AcbSh (DV: 5.5 to 7.5 mm from

the dural surface) were recorded with glass microelectrodes (average impedance of 6–10 MΩ) filled with 0.5M sodium acetate solution containing 2% pontamine sky blue (Sigma-Aldrich). Population spontaneous activity was determined in predetermined recording tracks separated by 200 μm and the activity of each neuron was recorded for 5 min. Single-unit neuronal activity was filtered (bandpass 0.3–5 kHz) and individual action potentials were isolated and amplified (MultiClamp 700B amplifier, Molecular Devices), digitized at 25kHz and recorded using a Digidata 1440 A and pClamp software (Molecular Devices).

Local field potential (LFP) signals were analyzed using NeuroExplorer (Nex Technologies). LFP were decimated to 1 kHz, and lowpass filtered (IIR Butterworth filter at 100 Hz; filter order set to 3). Subsequently, a spectrogram function was used to calculate the power of oscillations at frequencies between 0–100 Hz (window length 2 s; shift 0.5 s). Power values for a given frequency were averaged over the time of the recording epoch and normalized so that the sum of all power spectrum values equals 1. The total power was calculated by adding all the power values at frequencies between 0–59 and 61–100 Hz. Power values at 60±1 Hz were excluded from all the calculations. The bands were defined based on the following frequencies: delta 0.5-4 Hz, theta 4-7 Hz, alpha 7-14 Hz, beta 14-30 Hz, low gamma 30-59 Hz, high gamma 61-80 Hz, respectively. To perform histological analyses, at the end of recording sessions, DC current (20 mA for 15 min) was passed through the recording micropipette in order to mark the recording site with an iontophoretic deposit of pontamine sky blue dye.



Supplemental Fig.1: Schematic representation of the experimental timeline including the drug treatment and testing procedures.

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