UNIVERSITY OF GENOA

PhD Program in Experimental Medicine

Curriculum of Pharmacology and Toxicology

The acute effect of cannabis on ammonia dynamics: a potential new mechanism for THC in locomotor activity?

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The best ideas in science are almost always simple, elegant and unexpected.

Jeff Hawkins

SUMMARY

1. INTRODUCTION

1.1 Cannabis plant

Cannabis is one of the most controversial substances of the twenty-first century. The genus *Cannabis* is a member of the family plant Cannabaceae, and the primary cannabis species are *Cannabis sativa*, *Cannabis indica* and *Cannabis ruderalis*, which vary in their biochemical constituents.[1] Marijuana is the dry shredded green mixture of flower, stem, seeds and leaves of the *Cannabis sativa*. Botanical origins of cannabis are probably from Western and Central Asia. Cannabis has been cultivated since ancient times in Asia and Europe and then spread to America.[2]

The plant chemistry is very complex: it has over 421 different compounds, including over 60 phytocannabinoids, divided into 18 different classes of chemicals such as nitrogenous components, amino acids, hydrocarbons, carbohydrates, terpenes, and simple and fatty acids. In addition, there are more than 2,000 compounds that may be released after pyrolysis[3-5]. Δ^9-Tetrahydrocannabinol (Δ^9-THC, from now on THC) is the main psychoactive, as well as the most studied among the cannabis components.[6] Others major cannabinoids in *Cannabis sativa* plant are cannabidiol (CBD), cannabigerol (CBG), and cannabinol (CBN), that present less and different psychoactive effects from THC.

THC content in a plant can range from 0.4% up to 20%, depending on the strain, available light, soil type and pH, nutrients and fertilizers provided and time of harvest.[7-9] Over the past decades, the amount of THC in cannabis strains has been increasing steadily.[10]

The most potent early strain is the *sinsemillas*, a seedless marijuana from the inflorescences of the female plants. Other more common strains, known for their potency, are the *Purple Haze, Early Girl, Big Bud, Skunk, Superskunk* and *Hindu Kush*. The exposure to the plant occurs mainly through inhalation of cigarettes smoke (*joints)* or pipes (*bongs)* or through ingestion of different food products such as cakes, cookies, and candies.

Hashish, a potent cannabis derivative, is made from the resin collected from the tops of flowering plants. Often, hashish contains THC levels that exceed 10%.[11] Hash oil, another form of cannabis product, could contain even higher THC concentrations, up to 20%. A joint usually contains 500-1000 mg of plant material, corresponding to 15-30 mg of THC.[12,13]

Through the decades, the ideology around cannabis has taken a swinging journey from an illegal to a legal status. In the United States, in 32 states marijuana has been already legalized for medicinal use, in 9 states for recreational use, and decriminalized in another 14 states. In Italy, the therapeutic use of cannabis has been authorized since 2007 and nowadays personal possession and supply has been sensitively decriminalized compared to other drugs.[14]

1.2 Cannabis effects

Due to its many medicinal properties, cannabis has many uses since the ancient times.[15] The growing interest for the multitude of its potential medicinal uses has led to a significant pressure for legalizing medicinal cannabis and the research related to possible applications. To date, several pharmaceutical products containing different compositions and concentrations of THC and CBD have already received regulatory approvals (Sativex®, Marinol®, Cesamet®) or are under investigation as potential treatments of diseases such as anorexia-cachexia syndrome, chemotherapy-induced emesis, glaucoma and pain and spasticity in Multiple Sclerosis.[16, 17]

Moreover, cannabis is currently the most commonly used illicit drug on earth[18, 19]. Exposure to THC causes different effects varying from euphoria, palpitations, heightened sensory awareness, to altered time perception and coordination.[20] Occasionally, even low doses of THC, may precipitate a panic reaction, disrupt motor activities, alter nociception, and cause nausea and vomiting.[21, 22] Higher concentrations of THC could lead

to anxiety, impaired short-term memory, depersonalization, hallucinations, acute paranoid psychosis, tachycardia, orthostatic hypotension, conjunctival injection, incoordination, slurred speech, and ataxia.[20, 23]

Furthermore, long term cannabis use is associated with impairment of learning and memory, attention, working memory,[24, 25] executive functions, motor skills and verbal abilities.[26,27]

In addition, recent preclinical and clinical findings support the association between cannabis and amotivational syndrome.[28, 29] Since THC has proven to be able to disrupt learning, the reduced motivation has been suggested to be a pathway to impaired learning.[30] Interestingly, cannabis users show a reduction in striatal dopamine synthesis capacity,[31] with an inverse relationship to amotivation. Whereas dopamine sustains circuits of motivation, it was theorized that an impaired dopamine synthesis could be related to the loss of motivation in cannabis users.[32]

In the central nervous system (CNS), cannabis is known to act through two types of receptors. Cannabinoid receptors type 1 (CB1Rs) are distributed all over the central nervous system and associated with most of the CNS endocannabinoid signaling[33], while CB2Rs are mainly expressed in immune system cells. CB1R and CB2R are coupled to inhibitory $G_{i/o}$-proteins.[34, 35] THC activity on mesocortical and limbic systems, striatum and lateral prefrontal cortex, has been suggested to be the cause of the main clinical effects of cannabis.[36, 37]

There is an endogenous system of ligands for cannabinoid receptors (i.e., 2-arachidonoyl glycerol, anandamide, and N-palmitoylethanolamide) that act like neurotransmitters.[38] This system is crucial in various functions involving memory, emotions, movement, cell proliferation, and other important cell functions.[39] Cannabis, as well as other drugs of abuse, alters brain levels of endocannabinoids, therefore, part of the complexity of cannabis-mediated effects could to be due to its role on this endogenous system, proven to be involved also in processes of drug reward. Despite the massive research on neuronal cell cultures[40, 41], rodents,[42, 43] monkeys,[44, 45] and human brains, the mechanisms underlying cannabis effects and toxicity

are still not completely elucidated.[46]

1.3 Cannabis and ammonia

In a recent study, high concentrations of ammonia were detected in the cannabis smoke and vapor from the heated cannabis plant. [47] It has also been proved that stressing the cannabis plant by UV exposure, increases the content of THC and ammonia. [48] Back in time, De Pasquale et al. demonstrated how chronic parenteral administrations of cannabis resin in dogs can significantly increase blood ammonia concentrations.[49] A few years later, Ghoneim et al., proved that exposing rabbits to hashish smoke for one month, markedly increased their blood ammonia concentrations.[50] Moreover, inhaled ammonia released during smoking has been shown to be quickly absorbed through the lungs into the systemic circulation, where it diffuses passively through the blood brain barrier (BBB).[51]

The ammonia dynamics within the central nervous system (CNS) has been widely studied in several diseases, among all the hepatic encephalopathy and acute liver failure. Although the exact pathophysiology of hyperammonemia is still matter of debate, one recognized factor is that increased brain ammonia triggers its own detoxification by glutamine synthesis from glutamate by astrocytic glutamine synthetase (GS) enzyme.[52,53]

The glutamine produced in astrocytes is normally used as source of energy or, is converted into glutamate by the neuronal glutaminase (GA), with the release of ammonia (Figure 1).[54]

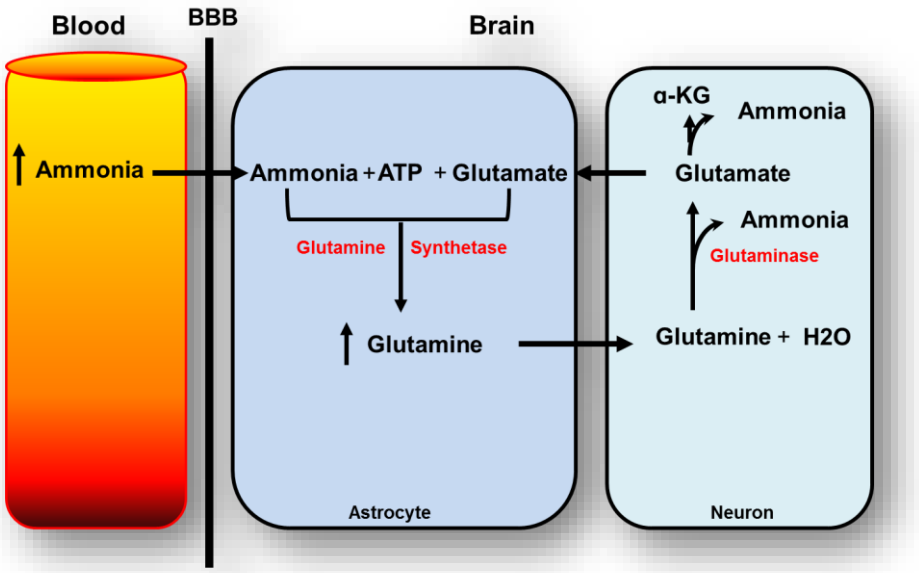

Figure 1. Representative scheme of ammonia cycle in blood, astrocytes and neurons. Ammonia inhaled with cannabis smoke diffuses into the systemic circulation and then passively through the blood brain barrier (BBB). Ammonia is included into glutamine by astrocytic glutamine synthetase. Astrocytic glutamine can be shuttled to glutamatergic neurons where it is converted into glutamate by glutaminase.

In the astrocytes, part of the brain glutamine can be replaced with neutral amino acids such as tryptophan, phenylalanine, and tyrosine from the blood.[55, 56, 57] Under normal conditions brain GS operates at near maximum capacity.[55, 58, 59]

Indeed, under physiological conditions, the ammonia released from neurons in the glutamate–glutamine cycle (during glutamate synthesis from glutamine) is used in same amounts for the synthesis of glutamine from glutamate in astrocytes, with no net ammonia production. In conditions of ammonia excess instead, the main mechanism of detoxification is the glutamate conversion into glutamine that, accumulating within the astrocytes may interferes with mitochondrial function and increase the production of free radicals. [25] or decreasing glutamate levels, interfering with neuronal transmission. [60, 61]

Furthermore, increased glutamine can produce an enhancement in

brain tyrosine and tryptophan concentrations, promoting consequently dopamine and serotonin synthesis (Figure 2).[62]

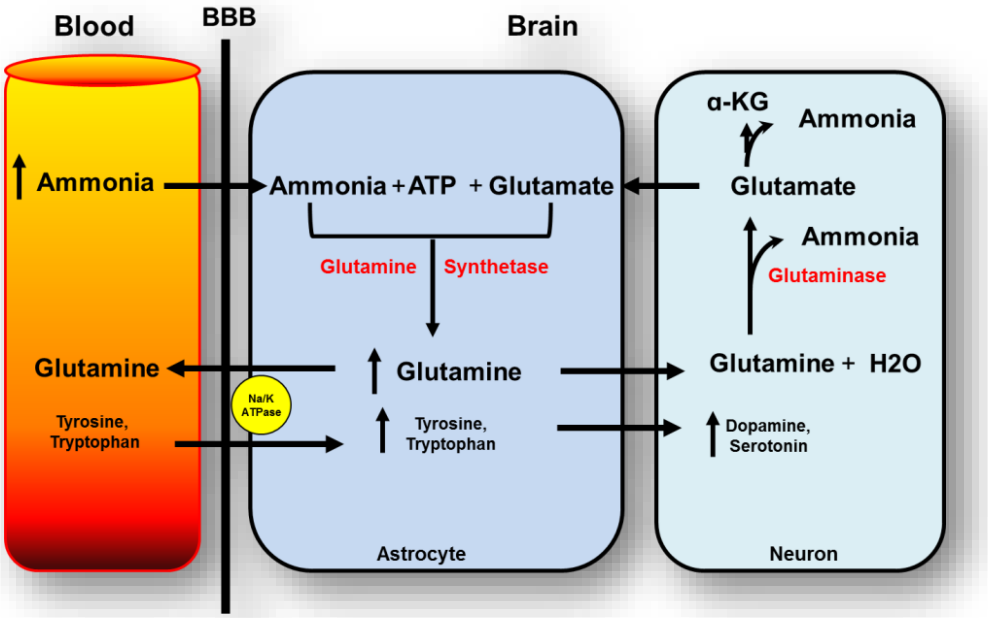

Figure 2. Representative scheme of ammonia cycle in blood, astrocytes and neurons. Glutamine synthetized in astrocytes can be exchanged with tryptophan, phenylalanine, and tyrosine from the brain to the blood and consequently promotes dopamine and serotonin synthesis.

Several i*n vivo* and *in vitro* studies reported that high ammonia concentrations in the brain have toxic effects on neurons, astrocytes and mitochondria, but how ammonia brings changes in these types of cells is not totally understood.

The critical factor in ammonia-induced cell impairment seems to be the oxidative/nitroxidative stress. One consequence of oxidative stress is the induction of the mitochondrial permeability transition, a Ca^{2+}-dependent process associated with a collapse of the inner mitochondrial membrane potential due to the opening of the permeability transition pore. Such membrane potential collapse leads to mitochondrial dysfunction, energy failure and additional free radical production.[63]

Secondary ammonia-mediated alterations are changes in neuronal

firing patterns,[64] activation of glutamatergic signaling[65] and swelling of astrocytes.[66] This last condition is also a major component of the brain edema associated with hyperammonemic state.

2. AIM OF THE THESIS

Either if marijuana is used for medicinal or recreational purposes, novel therapeutic targets that can effectively treat cannabis-related side effects are urgently needed and a better understanding of the mechanisms underlying cannabis effects seems crucial.

For this reason, our aim is to study the potential link to cannabis and ammonia, a new potential target to treat cannabis side effects. In order to do this, we quantified how cannabis is able to vary ammonia concentrations over time in healthy cannabis users and mice. Moreover, we aim also to investigate the causal relationship between cannabis-induced transient hyperammonemia and acute changes in locomotor activity at a neuronal level.

3. MATERIALS AND METHODS

3.1 Clinical Study

The study protocol was approved by the National Institute on Drug Abuse Institutional Review Board and all participants provided written informed consent. Clinical procedures took place at the NIDA – IRP, Baltimore, USA (ClinicalTrials.gov Identifier NCT02177513).

3.1.1 Study design

The clinical trial was designed as double blind, placebo-controlled, randomized, four-session crossover study. For the main clinical trial, 28 healthy volunteers that used to smoke cannabis ≥2x/month were recruited. However, in this thesis only the effect of controlled cannabis administration on plasma ammonia concentrations in a subgroup of subjects (n=14) will be reported, due to the late inclusion of plasma ammonia concentration within the clinical parameters evaluated in the ongoing main study.

Inclusion criteria for the recruitment were: - males and females from 18 to 50 years of age; - consuming cannabis in the past 3 months; - one urine screening positive for cannabinoid for frequent cannabis smokers. Exclusion criteria were: - use of cannabis for medical purposes; - history of significant adverse events associated to cannabis; - current physical dependence on any drug other than cannabis, caffeine, or nicotine; - presence or history of clinically significant illness; - pregnant or nursing females. Women in child bearing age were required to use an accepted method of contraception.

Primary outcomes included subjective and objective assessments, performance on neurocognitive and motor tasks, and cannabinoid concentrations in whole blood, oral fluid, urine, dried blood spots, and breath.

During each of the four sessions, subjects were administered with a

placebo or active oral cannabis dose, followed by either placebo or active smoked or vaporized cannabis. Only one active and one placebo dose were administered in each dosing session. Possible combinations were: active brownie and placebo vaporized, or active brownie and placebo cigarette, or placebo brownie and active vaporized, or placebo brownie and active cigarette or two placebo doses [placebo brownie and placebo vaporized, or placebo brownie and placebo cigarette]. The order for all four sessions was randomly assigned.

Participants consumed the oral, followed by the smoked or vaporized dose over 10 min. During the session, before the administration (time point 0) and at 2, 4, 6, 8, 10, 15, 30, and 90 min after the beginning of cannabis administration, blood samples were collected for the determination of plasma ammonia and blood THC levels.

The four sessions were schedule with a minimum interval of 72 hours, and, when not hospitalized, the patients were allowed to use cannabis between sessions.

3.1.2 Drugs

Natural cannabis inflorescence and placebo (containing 0.001 mg THC) were provided by the NIDA pharmacy. For the routes of administration by inhalation (smoke and vapor), cannabis doses (54 mg THC) were prepared as a cigarette, or the same quantity was extracted and delivered via a Volcano® vaporizing device (Storz and Bickel, Germany) [67]. For the oral doses, brownies were prepared following the Duncan Hiness Double Fudge® cake-like brownie instructions: the contents of an active or placebo cigarette were ground, baked for 30 min at 121 °C in aluminum foil, and mixed into equal portions of batter in a muffin tin.

3.1.3 Procedures

During each session, ten blood samples were collected in two

aliquots 0, 2, 4, 6, 8, 10, 15, 30, and 90 min after the beginning of cannabis administration in order to measure plasma ammonia concentrations and THC blood levels.

Plasma ammonia concentration. All the samples were collected in pre-cooled heparinized tubes and immediately centrifuged at 3000g (4°C) for 3 min. Plasma was collected at 4°C and ammonia concentrations were assayed with a commercial colorimetric assay kit (BioVision Inc., Milpitas, CA) according to the manufacturer's instructions. Every sample was assayed in duplicate. Ammonia concentration was detected with a Sunrise™ microplate absorbance reader (Tecan Group Ltd., Männerdorf, Switzerland). The inter-assay coefficient of variance of controls was <10%.

THC blood concentration. Blood samples collected at different time points were processed according to a previously published method. [68] Briefly, 100 µL of human blood was deproteinized with acetonitrile and cannabinoids were extracted from the supernatants using disposable pipette extraction WAX-S tips (DPX Labs, Columbia, SC, USA). An aliquot of the resulting organic phase was diluted with aqueous mobile phase, centrifuged, and injected into the Liquid chromatography tandem-mass spectrometry (LC-MS/MS) system. Linear ranges were 0.5–100 µg/L. Intra- and inter-day imprecision were 2.4–8.5%, and the accuracy was 88.9–115%.

3.2 Animal Study

3.2.1 Animals

All experimental protocols were conducted in accordance with U.S. National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and with the approval of the National Institute on Drug Abuse Animal Care and Use Committee. All the *in vivo* electrophysiology

experiments were carried out in accordance with the United States National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee. For all the experiments, one to six-month-old male C57BL/6J (wild-type, Strain 664, The Jackson Laboratory, ME, USA) mice were used. Prior to each procedure, mice were group housed in temperature and humidity-controlled rooms with *ad libitum* access to water and rodent chow (PicoLab Rodent Diet 20, 5053 tablet, LabDiet/Land O'Lakes Inc., MO, USA) on a 12 h light/dark cycle.

3.2.2 Drugs

Natural THC was provided by the NIDA pharmacy and prepared for injection by adding THC to TWEEN® 80 (Sigma Aldrich, St. Louis, MO) and 0.9% saline 1:1:18 (v:v:v). [69] The total volume injected ranged between 0.25 mL and 0.35 mL depending on the THC concentration and the animal weight.

The CB1 receptor neutral antagonist NESS 0327 (Cayman Chemicals, Ann Arbor, Michigan) was dissolved in a solution of TWEEN® 80, DMSO (Sigma Aldrich, St. Louis, MO) and 0.9% saline 1:2:7 (v:v:v).[69]

3.2.3 Open Field Test

Effect of THC on spontaneous locomotor activity*.* In order to determine which THC dose could cause a significant reduction in spontaneous locomotor activity, three groups of mice (n=32 each, n=8/each subgroup) were used. The first group was treated with THC 0 mg/kg (vehicle control: TWEEN 80® and saline), the second with a low dose of THC (3 mg/kg) and the last one with a high dose of THC (10 mg/kg). Spontaneous locomotor activity (distance traveled) was observed for 5 minutes before the THC/ vehicle intraperitoneal (i.p.) injection and 1-, 3-, 5- and 30-min post-administration (timepoint 0), depending on the subgroup (Fig. 4). For every

main group, we used four subgroups (n=8 each) for different time of euthanasia (1-, 3-, 5- and 30-min post dose).
Each mouse was placed in a plexiglass open field (OF) arena (32x32 cm) and the distance travelled was recorded with a video-tracking system and then analyzed with a VersaMax software (AccuScan Instruments Inc., Columbus, OH).

Effect of CB1R antagonist NESS 0327 in reversing the effect of THC on locomotor activity. One group of mice (n=15) was administered with NESS 0327 (i.p., 0,1 mg/kg) 30 min prior THC/vehicle injection (n=7-8/treatment, respectively), then animals were placed in a plexiglass OF arena and the entire session was video recorded for 5 minutes before and up to 20 min after THC injection. Data were analyzed with AnyMaze software (Wood Dale, IL).

Identification of the best dose of ammonium acetate to mimic THC effect on locomotor activity. Four groups of mice were used for this experiment (n=7 each). Mice were placed in a OF arena, distance travelled recorded for a 5-minute baseline, then treated with i.p. injections of vehicle control (distilled water), NH_4Ac 1 mM/kg, 3 mM/kg or 5 mM/kg. After the injections, mice were replaced in the arena and movements recorded for 20 minutes. Data were analyzed with AnyMaze software (Wood Dale, IL).

3.2.4 Ammonia and THC quantification

Euthanasia, blood collection. Following cervical dislocation, mice (8 animals/subgroup) were euthanized by rapid decapitation at 1, 3, 5 and 30 min. Trunk blood was collected in precooled (4 °C) heparinized tubes and centrifuged immediately [3000g (4 °C) x 3 min]. Collected mouse plasma was divided into 2 aliquots, one was stored at −20 °C for the determination of THC levels and the second one was used immediately for the colorimetric ammonia assay. Moreover, immediately following

decapitation also liver, prefrontal cortex (PFC), striatum (ST) and cerebellum (Cere) were dissected on ice water (4 °C) under a light microscope and transported on dry ice to a −80 °C freezer until assayed for ammonia concentration and enzymatic activity.

Blood and Plasma THC quantification. Mouse plasma samples (20 µL) were processed and THC quantification was performed as described above for the human blood samples.

Ammonia determination in plasma, liver and brain tissues. Mouse plasma (25 µL) was deproteinized with an equal volume of 8% perchloric acid and centrifuged at 4000g (4 °C) for 5 min. Specimens were neutralized with 2 M potassium bicarbonate and re-centrifuged at 4000g (4 °C) for 10 min prior to the analysis. Following the last collection, specimens were analyzed using a commercial ammonia colorimetric assay kit (Abcam®), following the manufacturer's instructions as mentioned above.

Brain and liver tissues were homogenized in 20 times w/v of ice-cold ammonia kit buffer (Abcam®) and centrifuged at 4000g (4 °C) for 5 min and processed as above.

We performed the experiments for ammonia quantification in the afternoon (between 1-6PM) to reduce the physiological variability in plasma ammonia concentration during the day.[70]

3.2.5 Glutamine synthetase and glutaminase enzymatic activity

To assess the glutamine synthetase (GS) enzymatic activity, brain tissues were homogenized in 20 times (w/v) of 60 mM imidazole-HCl buffer (pH 7.5) containing EDTA and spun at 1000g for 10 min at 4°C. GS enzymatic activity was assessed as described by Momosaki et al.[71]: supernatant was incubated with a 60 mM L-glutamine, 15 mM hydroxylamine HCl, 20 mM Na-arsenite, 0.4 mM ADP, 3 mM MnCl2, and 60

mM imidazole-HCl buffer solution, for 15 min at 37ºC, 1:10 v:v. The reaction was stopped by adding 1:1 v:v of a solution of 0.05 M trichloroacetic acid, 0.16 M HCl and 1.18 M $FeCl_3$) and incubated for 5 min at 37°C. Absorbance of the product of the reaction, the glutamyl hydroxamate, was assayed with a Sunrise™ microplate absorbance reader at 540nm. GS activity is expressed as mM of γ-glutamyl hydroxamate/min/mg of proteins. To assess the protein content a Pierce™ BCA Protein Assay Kit was used (ThermoFisher®, Halethorpe, MD).

To quantify the glutaminase (GA) enzymatic activity, brain tissues were homogenized in 20 times of buffer containing 25 mM Tris–HCl, 0.2 mM EDTA, 0.33 M sucrose. Samples were then solubilized with Triton (TX-100) and spun at 100,000g for 30 min.[68] The enzyme activity was assayed as previously described[71, 72]. Twenty-five µL supernatant was incubated for 1 h at 37°C with 35 µL of reagent mixture (Reagent 1, 100 mM potassium phosphate, 171 mM L-glutamine and 1.5 mM NH_4Cl). The reaction was stopped with the addition of 10 µL 10% tricarboxylic acid. Blanks and reagent 1 were incubated separately and then mixed after the addition of tricarboxylic acid. Samples and blanks were spun at 4,000g for 10 min. A 5 µL aliquot was add to 150 µL of a second reagent (0.2 M potassium phosphate, 72 mM mercaptoethanol in ethanol and 186 mM o-phthalaldehyde in ethanol). Samples were incubated at room temperature for 45 min in the dark. The absorbance was measured at 405 nm and compared to the absorbance generated by a standard curve of NH_4Cl treated with the reagent 2. GA activity is expressed as mM of ammonia produced/min/mg of proteins as previously reported.[71]

3.2.6 Functional imaging of neuronal activity of dorsolateral striatal neurons

Stereotaxic viral injections and GRIN lens implantation. For GRIN lens implantation surgeries, six to eight-week-old wildtype mice were used. Each animal was unilaterally implanted in either the left or right

hemisphere. Mice were anesthetized with isoflurane and placed onto a stereotaxic apparatus (David Kopf Instruments, CA, USA). After exposing the skull by a minor incision, a small hole (< 1 mm diameter) was drilled for virus injection and subsequent GRIN lens implantation. First, a beveled 25-gauge needle was inserted into the hole to create a guide path for the lens (needle: bregma, +0.75 mm; midline, ±1.90 mm; dorsal surface, −2.55 mm). Next, an adeno-associated virus (rAAV2.9/CAG.GCaMP6s.WPRE.SV40, titer: 1.34×1013 genomic copies/ml; University of Pennsylvania Gene Therapy Program Vector Core, PA, USA)[73]was injected (100 nl; rate: 30 nl/min) into the dorsolateral striatum (injection: bregma+0.75 mm; midline, ±1.90 mm; dorsal surface, −2.70 mm) by a pulled glass pipette (20−30 μm tip diameter) with a micromanipulator (Narishige International USA Inc., NY, USA) controlling the injection speed. After injection, either a GRIN lens (ILW-050-P146-055-NC; Go!Foton Corporaton, NJ, USA) or a cannula (MGC_560/610_5.2_v2.1; Doric Lenses Inc., QC, Canada)[74] was lowered into position above the injection site (lens: bregma+0.75 mm; midline, ±1.90 mm; dorsal surface, −2.20 mm). A head bar was attached to the skull surface with cyanoacrylate and dental cement (C&B Metabond Adhesive Cement, Parkell Inc., NY, USA) was spread around the lens or cannula and inside the head bar to hold everything in place. A final layer of black dental cement (Contemporary Ortho-Jet, Lang Dental Manufacturing Company Inc., IL, USA) was applied around the lens or cannula on top of the previous layer of cement. Two layers Parafilm were used to cover the GRIN lens or cannula, and the inside of the head bar was filled with Kwik-Sil (World Precision Instruments, FL, USA). After surgery, mice were individually housed.

Intraperitoneal catheter placement. Three days before the imaging experiment, mice were implanted with custom made catheters (Micro-Renathane® Tubing – Braintree scientific, Braintree, MA) for i.p. infusion. Inhalation anesthesia was maintained throughout surgeries with isoflurane via an E-Z Anesthesia brand vaporizer (E-Z Anesthesia, Palmer, PA). The incision site was sterilized with iodine and isopropyl alcohol. A small

abdominal incision of about 5 mm was made through the skin and peritoneum to implant the flexible tube. The tube was fixed to the internal abdominal wall with silk sutures to maintain the placement. The catheter terminals were guided with a trocar under the skin and exposed on the back of the mice, between the shoulders, to allow injections under the two-photon endomicroscopy setup.

Two-photon fluorescence endomicroscopy recording. We obtained three-dimensional resolution images of dorsolateral striatal neurons across sessions by using two-photon fluorescence endomicroscopy in awake, head-fixed mice. We used singlet (ILW-050-P146-055-NC; Go!Foton Corporation) GRIN lenses for tissue imaging in mice. The singlet lens has a working distance of approximately 130 μm on the object side. The singlet lens designs were previously described. [74] The numerical aperture (NA) was 0.5, which provides sufficient three-dimensional resolution for functional imaging of neuronal cell bodies and processes. The GRIN lens was incorporated into a two-photon fluorescence microscope equipped with a 5× air objective of 0.16 NA (Zeiss), which generated the initial focus of the excitation light (of 0.2 NA, to match the 0.19 NA of the GRIN lens on the image side) to be relayed by the GRIN lens to the sample side. The two-photon fluorescence signal was collected and transported back to the microscope by the GRIN lens and detected with a photomultiplier tube (PMT; H7422; Hamamatsu Corporation, NJ, USA). A Ti:Sapphire femtosecond oscillator (Mai Tai HP; Spectra-Physics, CA, USA) tuned to 940 nm was used as the excitation light source for all experiments. Eight weeks after surgery, the mice were habituated to both the head-restraint and the *in vivo* imaging setup. Such habituation consisted of escalating durations from 5–40 minutes over a period of 3–5 days. ScanImage 3.8 (Vidrio Technologies LLC, VA, USA) was used to collect *in vivo* imaging recordings at 1.5 Hz for all mice. Laser power was set to 10% (i.e. 45–48 mW) and it was consistent among all recordings to avoid brain tissue damage by heat. The acquisition speed was limited to 1.5 Hz by the

scanning stage galvanometer.

Functional imaging analysis. For the analysis of the two photon calcium imaging data, the Calcium Imaging Analysis (CaImAn) package[75] and Non- Rigid Motion Correction (NoRMCorre)[76] package were used in MATLAB R2018A (The MathWorks Inc., MA, USA). First, imaging recordings were motion corrected using the NoRMCorre code. Motion corrected recordings were then manually screened for frames containing aberrations due to motions that could not be corrected, and such frames were noted. A custom written MATLAB script aided in this selection of bad frames by giving the frame numbers where there was more than a 4 times standard deviation difference between frames. However, the script was only used as guidance and all the frames were manually checked. Next, using the CaImAn package's pipeline, regions of interest (ROI) are determined in the recordings using a constrained nonnegative matrix factorization (CNMF) approach finding the best spatial and temporal components explaining the observed fluorescence. This algorithm deals with heavily overlapping and neuropil contaminated movies, resulting in a fluorescence per ROI with the background signal subtracted. After extraction of the fluorescence per ROI, recordings over days were manually aligned to be able to track the same ROI over days. However, due to fluctuations in virus expression and recording in slightly different Z-planes not every ROI could be found in all sessions, resulting in the exclusion of this subset of ROIs. Data were displayed as heatmaps, single traces and integral of $\Delta F/F$.

Immunohistochemistry for functional imaging animals. After the recordings, to confirm the correct positioning of the lenses and virus transductions, immunohistochemistry assays were performed on mice brains (data not shown). Mice were deeply anesthetized with isoflurane and transcardially perfused with 1× PBS followed by 4% PFA in 1× PBS. Head bars and GRIN lenses were carefully removed to avoid damaging brain tissue. Whole brains were removed and post-fixed in 4% PFA/PBS for 2 h

at 4 °C and subsequently transferred to 1× PBS for storage at 4 °C until further processing. Coronal brain sections (50 µm thick) containing the striatum were collected in 1× PBS using a vibrating tissue slicer (vibratome; Leica VT1200), and freely floating slices were immunostained for green fluorescent protein (GFP). Sections were incubated with a blocking solution of 1× PBS/0.2% Triton X-100 (PBT) plus 5% normal donkey serum (NDS) for 1 h. Sections were then incubated with the primary antibodies chicken anti-GFP (1:1000 GFP-1020; Aves Labs, OR, USA) in PBT/2% NDS overnight at 4 °C. After rinsing 4 × 10 min in 1× PBS, sections were incubated for 2 h with secondary antibody donkey anti-chicken-Alexa Fluor 594 (1:500; Invitrogen) in PBT/2% NDS at room temperature and washed with 1× PBS (4 × 10 min). Sections were mounted with DAPI-Fluoromount-G aqueous mounting medium (Electron Microscopy Sciences) onto Superfrost Plus glass slides (VWR International). Images were taken with an AxioZoom.V16 fluorescence microscope.

3.2.7 Extracellular recordings of dorsolateral striatal neurons

Microelectrode arrays (MEA). Custom-made microelectrode arrays (MEA) were obtained from Innovative Neurophysiology (Durham, NC). The MEAs had 16 x 35 µm tungsten electrodes. MEA were encased in dental cement (Industrial Grade Grip Cement, powder #675571, liquid #675572, Dentsply, York, OA).

Stereotaxic MEA implantation. At 8-12 weeks of age, mice were anesthetized with isoflurane via an E-Z Anesthesia brand vaporizer (E-Z Anesthesia, Palmer, PA). A craniotomy above the dorsolateral striatum was centered at bregma, +0.75 mm; midline, ±1.90 mm and was extended into a concentric square (bregma, ±0.3 mm; midline, ±0.3 mm). The MEA was slowly lowered -2.70 mm from the dorsal surface (from bregma) into the brain and secured to the skull using 2 anchor screws (MF-5182 bone screws; Bioanalytical Systems, Inc, West Lafayette, IN), with the ground

wire wrapped around one screw and inserted 2 mm into the cerebellum. The array was then encased in a dental cement headcap (Industrial Grade Grip Cement, powder #675571, liquid #675572, Dentsply, York, OA).
Following surgery, the mice recovered for two weeks before starting the recordings.

Recording Hardware. *In vivo* electrophysiology recordings were performed using an Omniplex-DHP system from Plexon, Inc (Dallas, TX), with a motorized commutator from NeuroTek (Toronto, Ontario, Canada), with the IV fluid port used to feed in the patch cord to the mouse's head. The wideband signal was acquired at 40k Hz, with an analog high-pass filter of 7.5 Hz, and a 7.5 kHz low pass filter before the signal was digitized at the head stage. The continuous spike data was extracted from the wideband signal by applying a 4-pole 500 Hz Butterworth high pass filter and a 4-pole 3000 Hz Butterworth low pass filter. Common median referencing was used for all recording sessions.

Data Processing and Analysis. Plexon OfflineSorter 4 was used to process and separate identified units from each recording. Units from the same electrode were considered to be separate as long as the waveforms were significantly separated in 3D principal component space as tested by multivariate ANOVA ($p<.05$), with L-ratios <0.05 for each cluster.

3.2.8 Statistical analysis

Data are reported as mean ± SEM unless otherwise noted. Statistical analyses were performed using the Analysis ToolPak of Microsoft Excel 2016 (Microsoft Corporation, WA, USA) and GraphPad Prism (Prism version 7, GraphPad software, La Jolla, California) or OriginPro v9.2 (OriginLab Corporation, MA, USA). Statistical significance (*p* values) for paired comparisons was determined by two-tailed Student's *t*

test, and $p < 0.05$ was considered statistically significant. Experimenters were blinded during the clinical trial but not for the animal study.

Relationship between mouse plasma or human blood THC concentrations and ammonia concentrations was examined using Pearson's correlation. Behavior data were analyzed with the Analysis ToolPak of Microsoft *Excel* 2016, ANY-maze (Stoelting Co., IL, USA), GraphPad Prism, and MATLAB R2017B. Data from calcium imaging and electrophysiological recordings were analyzed with MATLAB R2017B (The MathWorks Inc., MA, USA) and Prism (Prism version 7, GraphPad software, La Jolla, California.

4. RESULTS

4.1 Human Study

4.1.1 Demographics

The cohort for this pilot study consisted of 14 cannabis users with a mean (±SEM) age of 29.9 (±2.7). The sample had 10 males and 4 females, 11 African Americans and 4 Caucasians. Seven subjects reported current nicotine smoking and 14 were alcohol drinkers but none met alcohol abuse or dependence criteria.[77] The subjects reported starting cannabis use around ages 14.6 (±0.9). The cumulative duration of cannabis use was 9.8 (±2.1) years; the average number of joints/week was 61.8 (±27.0) while the peak number was 86.1 (±29.7) joints/weeks.

4.1.2 Plasma ammonia concentration

Plasma ammonia concentrations increased significantly in subjects who received active cannabis compared to placebo (Figure 3.A). The baseline showed an important individual variability in ammonia concentrations, probably due to the confounding effect of the nicotine smoking, since ammonia is a natural component of tobacco leaves [78] and also used as an additive to commercial cigarettes.[79] Two-way ANOVA with time and route effects revealed a significant main effect of time [$F(8,256)=4.597$, $p<0.0001$], and route [$F(3,32)=4.339$, $p<0.05$], but no interaction between the two factor [$F(24,256)=1.044$, $p=0.4$]. Multiple comparisons demonstrated significant differences: placebo vs edible marijuana group at 30 min ($p<0.05$) and 90 min ($p<0.05$), and at 90 min placebo vs vaporized ($p<0.05$) and smoking routes ($p<0.05$). The concentrations of plasma ammonia positively correlated with the blood THC concentration (Figure 3.B, Pearson's $r=0.012$, $n=297$, $p<0.05$).

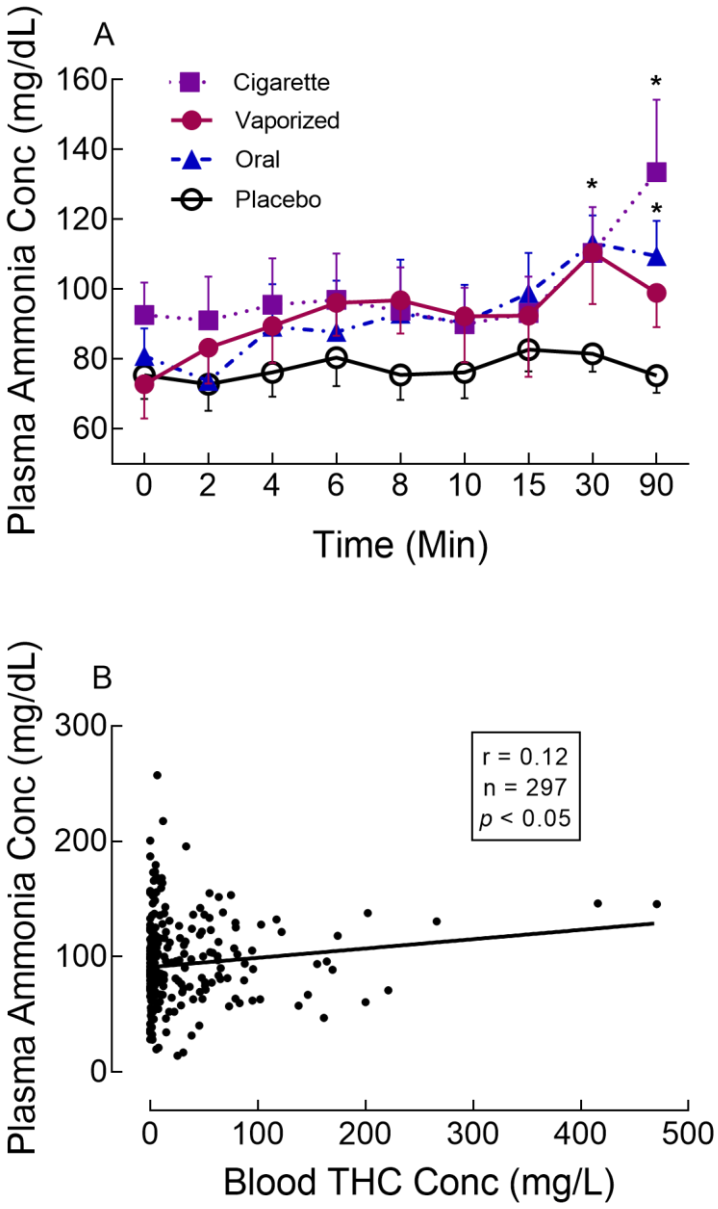

Figure 3. Cannabis administration increases plasma ammonia concentration. **A.** Plasma ammonia concentration (mean ± SEM) mg/dL in healthy volunteers administered with cannabis through different routes of administration versus placebo measured at baseline (0), 2, 4, 6, 8, 10, 15, 30, and 90 min post dose. **B.** Correlation between plasma ammonia concentration and blood THC concentrations at all the time points.

4.2 Animal Study

The pattern of delayed increase in plasma ammonia concentration observed in the human study could not be explained by simple diffusion of inhaled ammonia from the lung to systemic circulation. For this reason, we hypothesized that THC itself might be able to increase ammonia concentration in the brain, and this ammonia can then diffuse back through the blood brain barrier into the blood circulation, leading to the observed delayed increase in plasma ammonia concentration at 30 and 90 min after cannabis administration.

To test our hypothesis, in the animal study we administered THC instead of the cannabis plant which is known to contain ammonia.

4.2.1 Effect of THC on spontaneous locomotor activity

We found a reduction in the locomotor activity after the administration of both doses (3 and 10mg/kg) of THC compared to 5 min baseline (Δ locomotor activity). Two-way ANOVA with time and THC treatment (0 mg/kg, 3 mg/kg, and 10mg/kg) revealed a significant effect of time $F(34, 714)=45.34$, $p<0.0001$, treatment $F(2,21)=12.48$, $p<0.0001$, and interaction between the two factors $F(68, 714)=2.754$, $p<0.0001$. Post hoc *t*-test showed significant reduction in locomotor activity compared to 0mg/kg in the 3 mg/kg dose at 15, 22–25, 27 and 30 min and in the 10 mg/kg dose at 2, 5, 6, 9–15, 17–27 and 30 min post injection (Figure 4.A)

The overall Δ locomotor activity (Figure 4.B) was significantly reduced [($F=17.41$, $p<0.0001$) by one-way ANOVA], in both active THC dose groups: between 0mg/kg and 3 mg/kg [mean difference and 95% CI=58.0, 18.2–97.8, $p<0.001$] and 0mg/kg and 10mg/kg doses [mean difference and 95% CI=90.49, 56.0-125.0, $p<0.0001$] by Dunnett's multiple comparisons test.

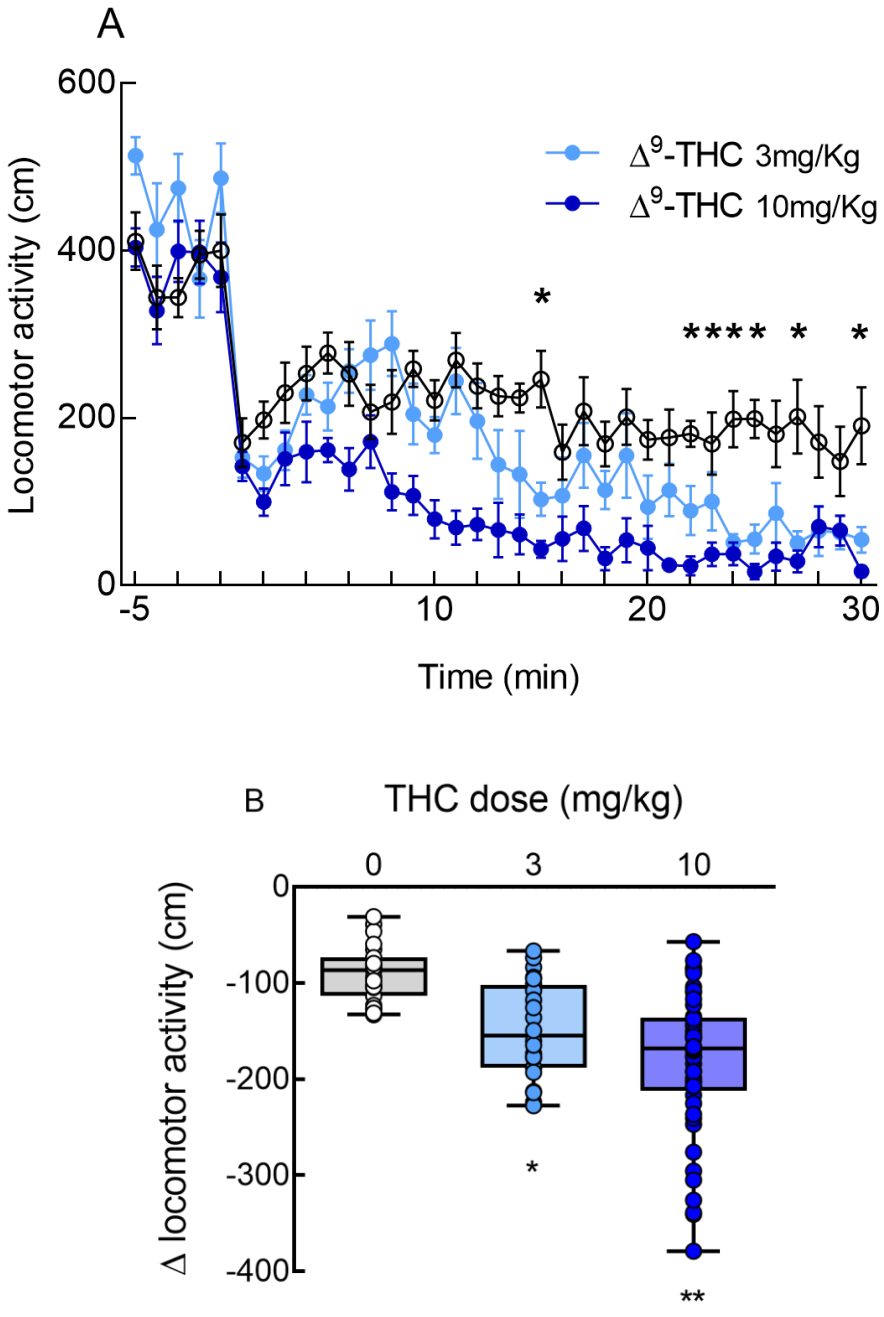

Figure 4. THC decreases spontaneous locomotor activity in mice. A. Locomotor activity (mean ± SEM) measured as distance travelled before and after THC injection at the timepoint 0 (n=8 for each group of treatment). **B.** Overall (at all the time points between 0 and 30 min) Δ locomotor activity for the 3 different doses. THC 3 and 10 mg/kg were compared to 0 mg/kg.

4.2.2 Effect of THC on plasma, liver and brain ammonia concentration

No significant difference among the first three time points (min 1, 3 and 5) was found, so data were pooled together. Within this early time window THC administration was not associated with changes in plasma ammonia concentration (F=0.679, *p*=0.5, Figure 5.A) and no correlation between plasma THC and plasma ammonia concentrations (r=-0.085, *p*=0.3, Figure 5.B) was found. However, comparing plasma ammonia concentrations between treatment groups at 30-min post injection showed significant difference [F(2,21)=3.682, *p* =0.042], driven mainly by the 3 mg/kg THC group [3 mg/kg vs.0 mg/kg: 0.13470.00 vs.0.12270.00, t=2.329, df=13, *p*=0.03, Figure 5.C]. Concentrations of plasma THC were not assessed at the latest time point.

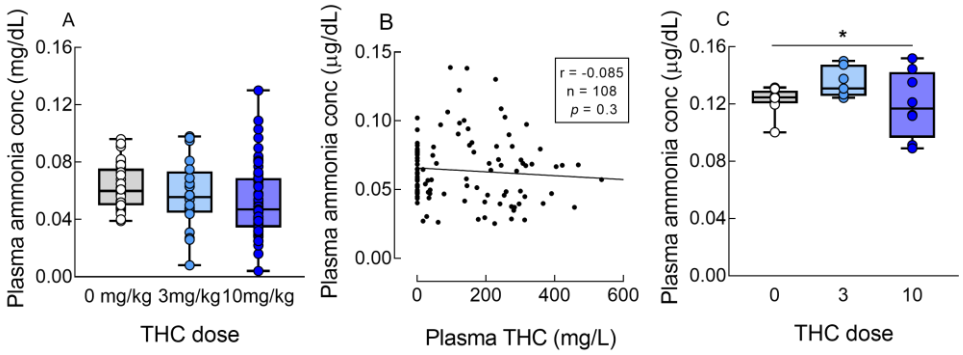

Figure 5. THC increases plasma ammonia concentration only 30 min post-injection. **A.** Plasma ammonia concentration at min 1, 3 and 5 (n=32). **B.** Correlation between plasma THC and plasma ammonia concentrations at min 1, 3 and 5. **C.** Plasma ammonia concentration at min 30 (n=8).

Liver ammonia concentration was not changed by THC (F=2.308, *p*=0.1, data not shown) at any timepoint.

Furthermore, we found a significant increase in ammonia level in the striatum of mice treated with THC (F=4.298, *p*=0.016, Figure 6.A) and

significant positive correlation (r=0.321, *p*<0.001, Figure 6.B) between striatal ammonia and plasma THC concentrations during the first 5 min. At the 30-min time point, was observed a 10-fold increase in striatal ammonia concentration [F(2,20)=4.043, *p*=0.033, Figure 6.C] induced by both THC doses [3 mg/kg vs. 0mg/kg: 0.24370.02 vs.0.02470.00, t=10.87, df=13, *p*<0.0001 and 10mg vs.0mg/kg: 0.08070.00 vs.0.02470.00, t=5.784, df=14, *p*<0.0001].

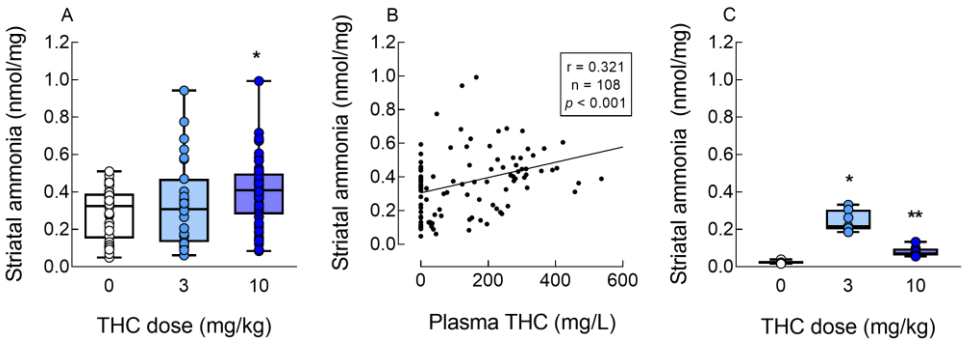

Figure 6. THC increases striatal ammonia concentration 5- and 30-min post-injection. **A**. Ammonia concentration in the striatum at min 1, 3 and 5 (n=32). **B.** Correlation between plasma THC and striatal ammonia concentrations at min 1, 3 and 5. **C.** Ammonia concentration in the striatum at min 30 (n=8).

On the other hand, we measured no changes in ammonia levels in PFC (F=1.617, *p*=0.2) or cerebellum (F=0.076, P=0.9), nor correlation between plasma THC and ammonia concentrations in either brain region [PFC (r=0.116, *p*=0.2), and cerebellum (r= -0.058, *p*=0.5)] during the first 5 min and at 30-min post injection [PFC [F(2,21)=0.1652, *p*=0.8], cerebellum [F(2,21) =1.068, *p*=0.3] were found.

4.2.3 Effect of THC on glutamine synthetase and glutaminase enzyme activity

We measured striatal GS and GA activity in three brain regions, such as striatum, cerebellum e prefrontal cortex (PFC), within the first 5 minutes after THC administration. We found significant reduction of GS activity (F=3.243, p<0.05) caused by the 10mg/kg dose (for control vs. THC 10 mg/kg = 28.1671.5 vs.23.1171.3, p<0.05, Figure 7.A) and negative correlation between plasma THC and striatal GS activity (r= -0.284, p<0.05, Figure 7B). We did not observe similar THC effect on GS activities in PFC (F=0.494, p=0.6) or cerebellum (F=0.929, p=0.4) and there was no correlation between plasma THC and GS activities in these brain regions (PFC: r=-0.143, p=0.2, Figure 5D, cerebellum: r=-0.199, p =0.13).

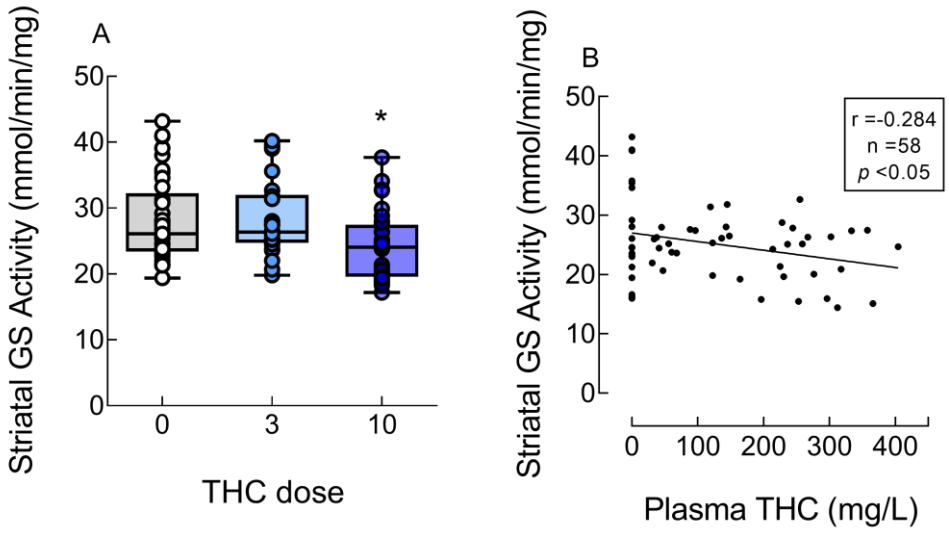

Figure 7. THC decreases glutamine synthetase (GS) activity in the striatum. A. GS activity in the striatum at min 1, 3 and 5 (n=32). **B.** Correlation between plasma THC and striatal ammonia concentrations at min 1, 3 and 5.

GA activity did not show either any significant changes (F=0.146, p=0.8), in PFC (F=0.047, p=0.9), and cerebellum (F=1.348, p=0.2) or correlation with plasma THC concentration in the striatum (r=0.023, p=0.8), PFC (r=0.024, P=0.8), and cerebellum (r=0.259, p=0.063).

4.2.4 Effect of CB1R antagonist NESS 0327 in reversing the effect of THC on locomotor activity

To better understand the involvement of CB1 receptor in the effect of THC on locomotor activity, we pretreated a group of mice (n=15) with the potent neutral CB1 receptor antagonist NESS 0327 (i.p., 0.1 mg/kg) 30 min before THC injection in order to block the receptor and eliminate the THC effect promoted through this type of receptor.

NESS 0327 successfully reversed the overall locomotor effects of THC (THC vs. Control: **p*<0.0001, Figure 8. A) at 20 min post THC injection. Remarkably, NESS 0327 did not reverse the THC effect within the first 5 minutes post-injection (THC Vs. NESS: **p*<0.0001; THC+NESS vs. Control: ***p*<0.005, Figure 8. B), suggesting that during this early time window hypolocomotion could be not entirely mediated by CB1receptors.

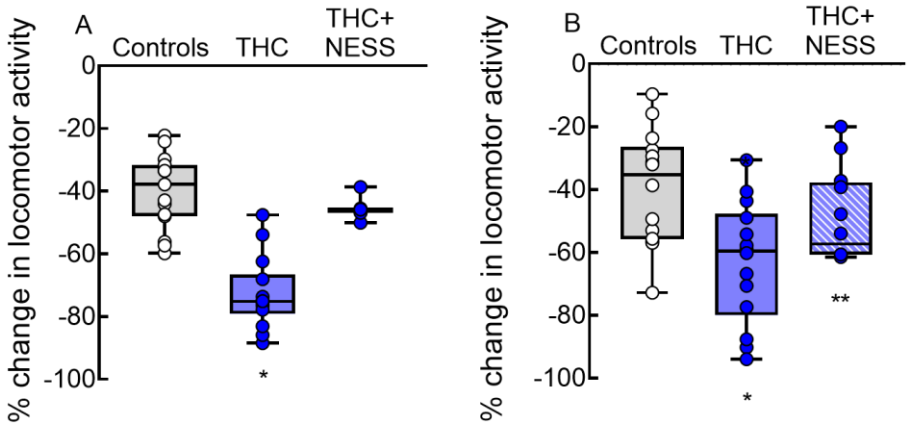

Figure 8. CB1 receptor antagonist NESS 0327 neutralize the overall effect of THC on locomotion, but not within the first 5 min. A. Overall Δ locomotor activity (percentage change from baseline) at 20 minutes. One group of mice was administered with THC (10 mg/kg), a second group was pretreated with neutral antagonist of CB1R NESS 0327(0.1 mg/kg) 30 min prior injection of THC (10 mg/kg) and the third group was injected with vehicle control. **B**. Δ locomotor activity for the three different treatments at the first 5 min post THC injection compare to vehicle control.

4.2.5 Identification of the best dose of ammonium acetate to mimic THC effect on locomotor activity

To prove that part of the effect mediated by THC is determined by the region-specific increase in ammonia concentration, we first found the most appropriate dose of NH_4Ac able to produce effects on locomotor activity similar to 10 mg/kg of THC. NH_4Ac has been used in several studies to induce a hyperammonemic state.[80, 81] We used four groups of mice (n=7 each): vehicle control (distilled water), NH_4Ac 1 mM/kg, 3 mM/kg or 5 mM/kg. The distance travelled was recorded for a 5-minute baseline, then after the i.p. injections, mice were placed againin the arena and movements recorded for 20 minutes.

Our results showed that NH_4Ac was able to reduce the locomotor activity in a dose dependent manner. THC and NH_4Ac 3 mM/kg caused similar significant reduction of locomotor activity compared to the controls (One way ANOVA, Tukey's multiple comparisons: THC vs Controls: **p*<0.0001; NH_4Ac 1 mM/kg vs. Controls: *p*=0.0001, NH_4Ac 3 mM/kg vs. Controls: **p*<0.0001 NH_4Ac 5 mM/kg vs. Controls: *p*<0.0001, No significative difference between the effect of THC and NH_4Ac 3 mM/kg: *p*=0.88 *n*=8/group, Figure 9).

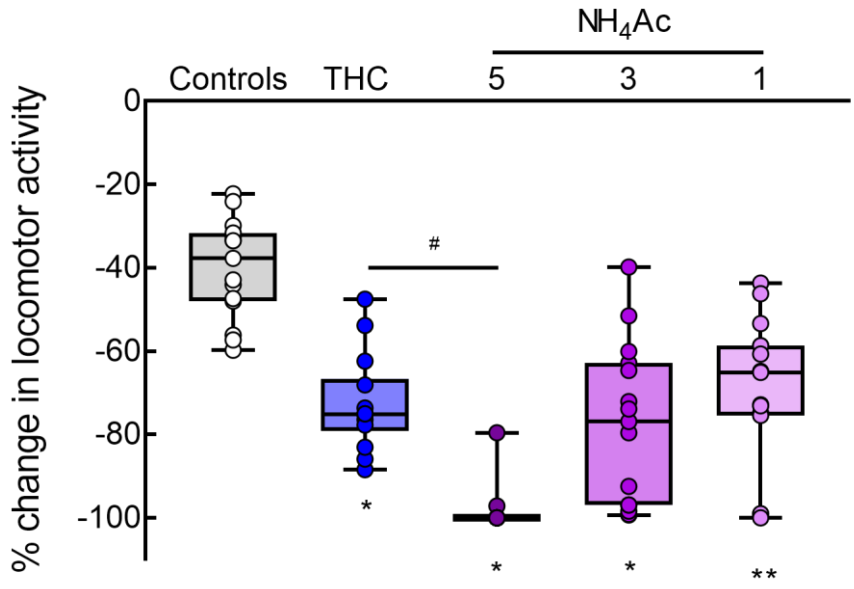

Figure 9. Ammonium acetate (NH_4Ac) mimics THC effect on locomotion. Comparison of the Δ locomotor activity (percentage change from baseline) at 20 minutes after administration of THC (10 mg/kg) and 3 doses of NH_4Ac (1, 3 and 5 mM/kg) compare to vehicle control (n=15/each group).

4.2.6 Functional imaging of neuronal activity of dorsolateral striatal neurons

In order to understand the direct effect of THC on the neuronal activity in the dorsolateral striatum and whether is comparable to NH_4Ac effect, we started a pilot study using *in vivo* endomicroscopy coupled with genetically encoded calcium indicators (GCaMP6s). This technique enables the analysis of fluctuations in the intensity of calcium-sensitive fluorophores, as an indicator of neuronal activity in awake, head fixed mice.[74]

We acquired functional images from dorsal striatum GCaMP6s neurons for each mouse (8 neurons, n=2 mice; unilateral) in sessions of 5-minute baseline, 20-minute after vehicle (TWEEN 80® and saline) injection followed by 20 min post injection of THC 10 mg/kg or NH_4Ac 3 mM/kg depending of the session. Mice were head-restrained during the experiment. Due to the setup, drugs were administered under the two-photon microscope in the dark, through i.p. catheter implanted 3-5 days before the first day of recording. Each mouse was exposed to THC or NH_4Ac with a week of time window to avoid potential confounding effects.

Our preliminary results (Figure 10) showed a decrease in the calcium transient over time (reported as the integral of the fluorescence compared to baseline, $\Delta F/F$) in the analyzed striatal neurons after i.p. injection of THC and NH_4Ac (two tailed paired t-test: controls vs. THC *$p<0.001$, Controls vs. NH_4Ac: **$p<0.05$)

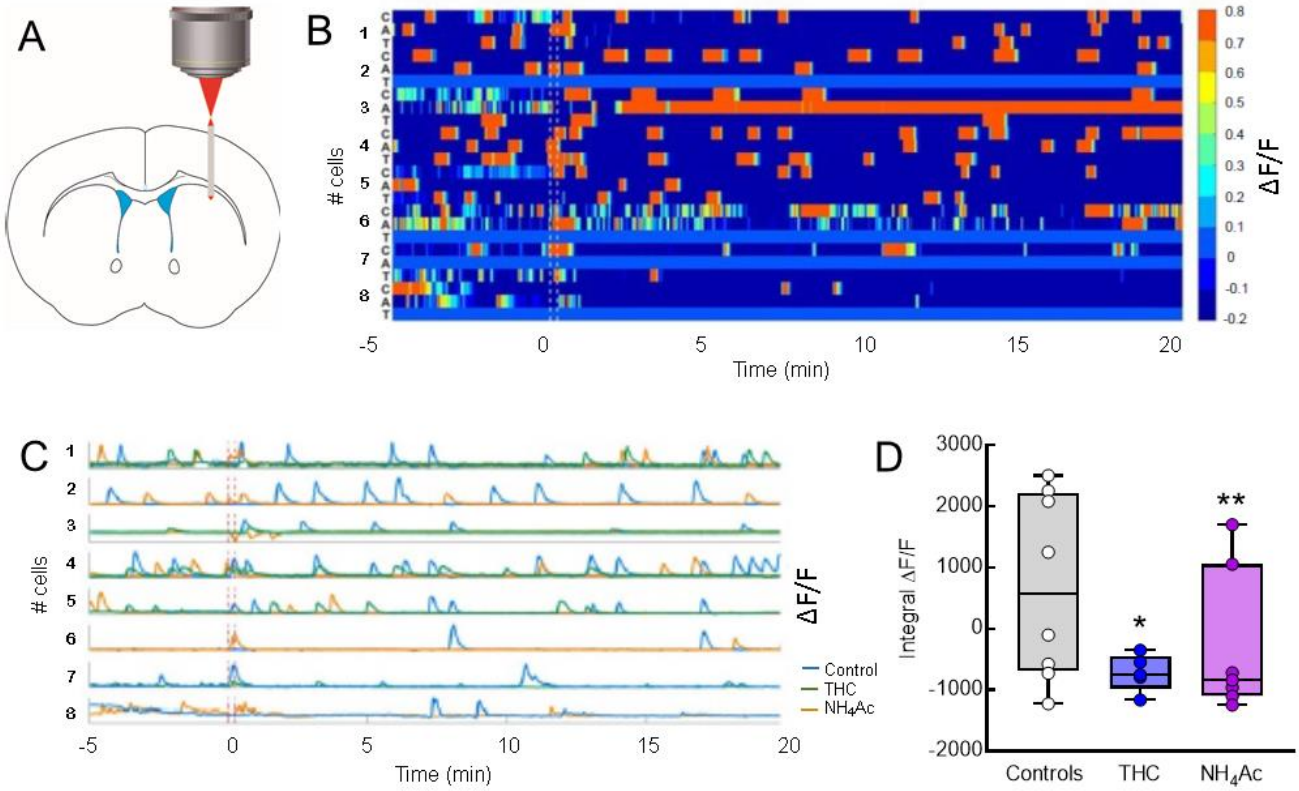

Figure 10. THC and NH_4Ac similarly reduce the neuronal activity in the striatum revealed by calcium imaging with 2-photon endomicroscopy in awake, head fixed mice. A. Schematic enlarged view of GRIN lens relaying the focus of the objective into a focus inside of the dorsolateral striatum. **B.** Heat maps for each cell (n=8, animals n=2) displaying the change in fluorescence (ΔF/F) over time after administration of vehicle control (C), ammonium acetate (NH_4Ac, A) and THC (T). Each row displays the activity of one cell, and red color denotes high activity while dark blue denotes low activity. **C.** ΔF/F calcium transient traces of neuron outlined in Figure 10.B for each of the 3 states. **D.** Box whisker plot of the integrals of neuronal activity during the 3 states, showing statistically significant reduction in activity after administration of THC (*p<0.001) and NH_4Ac (**p<0.05).

4.2.7 Extracellular recordings of dorsolateral striatal neurons

In order to analyze the direct effect of THC and ammonia on the

action potentials and firing rates of the dorsolateral striatal neurons, we started a second pilot study using *in vivo* electrophysiology on freely moving mice. All the *in vivo* extracellular recordings were conducted in collaboration with the laboratory of Dr. Dennis Sparta, Anatomy and Physiology Department, University of Maryland, Baltimore, MD.

The experimental design was similar to the one used for the *in vivo* calcium imaging experiment described above. We acquired extracellular recordings from dorsal striatum neurons for each mouse (13 neurons, n=2 mice; unilateral) in sessions of 5-minute baseline, 20-minute after vehicle (TWEEN 80® and saline) injection followed by 30 min post injection of THC (10 mg/kg) or NH_4Ac (3 mM/kg). Each mouse was exposed only once to the compounds to avoid potential confounding effects.

Preliminary results showed that both THC and NH_4Ac were able to reduce steadily the firing rate compared to vehicle (two-way ANOVA for repeated measures and Dunnett's test: THC, n = 8, $F(1,341)= 52.92$; $p<0.01$; NH_4Ac, n = 5,$p<0.001$; Figure 11)

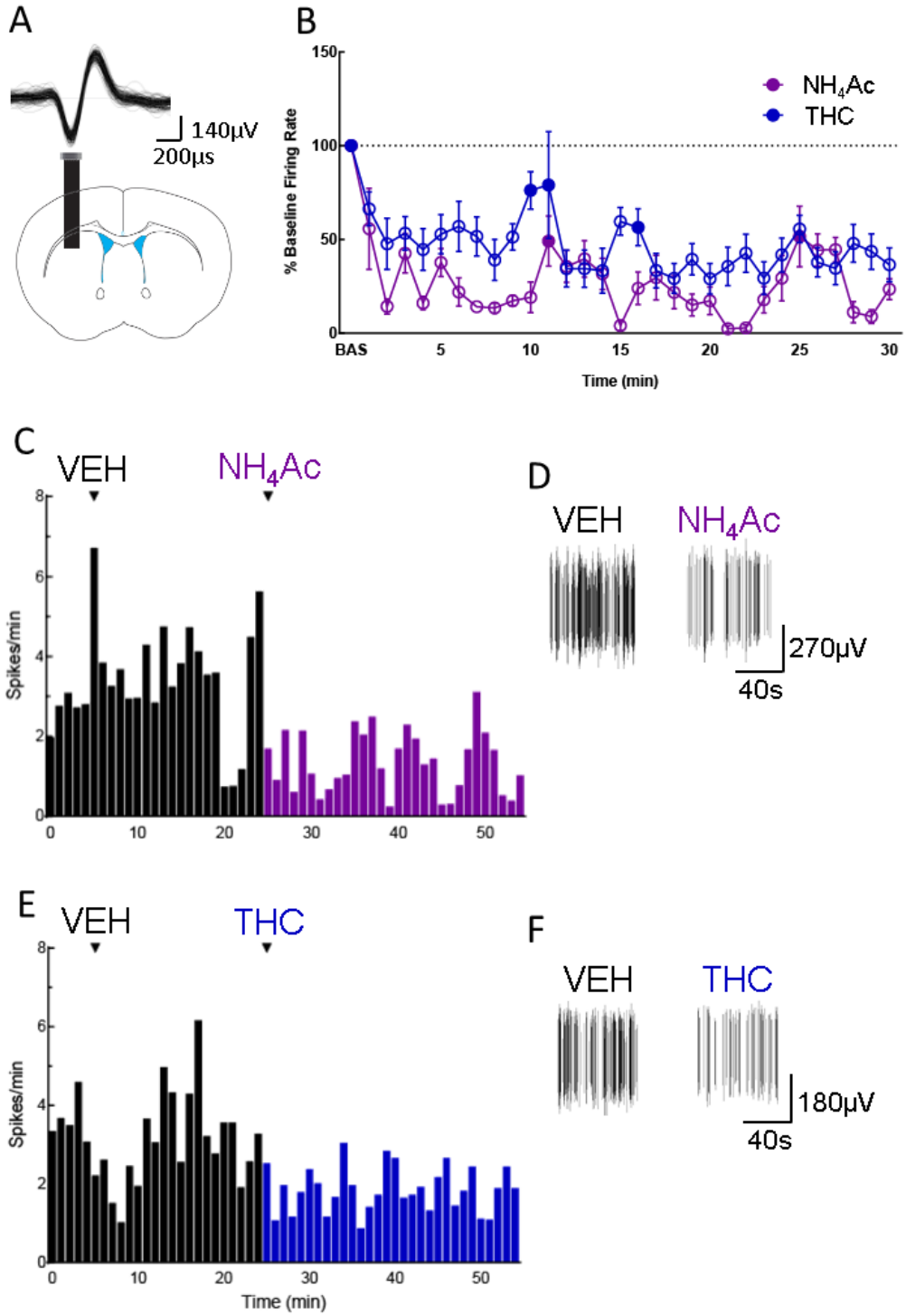

Figure 11. THC and NH_4Ac similarly reduce the neuronal activity in the striatum revealed by in vivo electrophysiology recordings in awake, freely moving mice. **A.** Schematic illustration of the electrode placement in dorsolateral striatum. **B.** Change over time curves displaying the effect of NH_4Ac and THC on the firing rate of dorsal striatal neurons (NH_4Ac, n=5;

THC, n=8). Results are presented as mean ± SEM of firing rate expressed as a percentage of baseline levels. Significant data points ($p<0.05$) were assessed with one-way ANOVA for repeated measures vs. baseline and are represented as un-filled circles in the graph. **C, E.** Representative firing rate histogram showing the decrease in discharge rate of an individual neuron following i.p. administration of NH_4Ac or THC. Arrowheads indicate the time of injection and the treatment administered. **D, F.** Representative traces of a dorsolateral striatal neuron before and after NH_4Ac or THC administration.

5. DISCUSSION

5.1 Cannabis and ammonia concentrations

We conducted our research with the aim to elucidate how cannabis can modify the concentration over time of ammonia after controlled administration, both in human cannabis users and mice. For the first time, we demonstrated that cannabis administration is able to increase plasma ammonia concentrations in cannabis users in a time- and route of administration-dependent manner.

Using a back translational approach, we demonstrated a significant effect of acute THC administration on striatal ammonia concentration associated with reduction in GS activity in wildtype mice. The choice of testing THC in the animal study instead of cannabis plant was made in order to avoid potential confounding effects, since THC ($C_{12}H_{30}O_2$) does not contain nitrogenous compounds in its chemical structure, while the cannabis plant contains and releases ammonia during heating or smoking[47, 48]

Interestingly, the clinical study revealed that cannabis administration though different routes of administration led to a different timing in ammonia detection in plasma: while smoked and vaporized cannabis significantly increased ammonia concentrations at 90 min, an increase in ammonia following the oral administration was observed at 30 and 90 min. However, despite the presence of ammonia in cannabis plant, such a delay from the onset of administration in the observed increase in plasma ammonia concentration, suggested a potential direct effect of cannabis in ammonia dynamics. Furthermore, after oral intake of the plant, the ammonia produced, could have caused only an increase in ammonia concentrations in the portal circulation and immediately detoxified in the liver by urea cycle, without causing an increase in systemic circulation.

This hypothesis was supported by our animal study results. In accordance with previously published data,[82] we found that administration of THC immediately leads to a temporary reduction in spontaneous

locomotor activity in mice. Then, we measured plasma, liver and brain tissue ammonia concentrations at three early time points (1, 3, and 5 min) and a late time point at 30 min, which correspond with the locomotor behavioral effect of THC. Our data showed that a single dose of THC has no effect on plasma or liver ammonia concentrations. However, we found a significant increase in striatal ammonia within 5 min post THC injection, with no significant changes in the other studied brain regions, and at 30 min. However, at 30 min, striatal ammonia concentration in each condition (THC 0, 3 and 10 mg/kg) resulted lower than at 1,3 and 5 min. This slight general increase and decrease in ammonia levels, could be explained with the stress induced by the injection in all the treated mice.

Ammonia assays were performed using a traditional colorimetric method, according to the manufacturer's protocol; we also followed strict specimen integrity guidelines by maintaining specimens at 4°C, excluding any hemolyzed specimens from analysis. Calibrators, blanks, in-house quality controls and specimens were analyzed in duplicate. Absorbance of assayed substances was averaged. Liver and brain tissues were maintained at an appropriate temperature during the collection and storage; all tissue samples were run under the same conditions and at the same time in order to minimize variability and allow valid comparisons. All these precautions allowed to obtain plasma ammonia values consistent with previously reported concentrations[57, 82] as well as variability in ammonia concentration in different brain regions also consistent with what reported in literature.[, 84, 85, 86, 87]

Interestingly, the increase in the striatal ammonia was coupled with a significant transient reduction in GS activity at 1 min, suggesting that THC suppresses striatal GS activity and increases brain ammonia concentration within minutes from acute THC injection, without mediate any change in plasma and liver ammonia concentrations. In support of our data on GS enzyme activity, a previous report demonstrated that prenatal administration of THC was able to reduce the astrocytic GS expression in mice.[88] Interestingly, striatal GS activity measured at 3 and 5 min or GS activity

measured in other brain areas (data not shown) did not show significant change, which suggests that the effect of THC on GS activity is transient and brain region specific. The underlying mechanism behind GS suppression is not clear. Glutamine accumulation was found to reduce GS activity.[59] Also the reduction in GS activity could stem from a direct effect of THC or indirectly through the reduction of ATP synthesis since GS is an energy demanding enzyme. Regardless of the mechanism, slight reduction in GS activity could have a noticeable effect on brain ammonia concentration given that the activity of brain GS is near maximum capacity.8[8, 89] Moreover, suppression of GS activity, theoretically, leads to accumulation of glutamate in the astrocytes and reduced capacity of astrocytic glutamate transporter that results in increased synaptic glutamate concentration, activation of neuronal NMDA receptors and potential neurotoxicity.[88]

An increase in ammonia concentration is known to induce NMDA receptor-mediated toxic effects in most animal species, altering many of brain functions. Hyperammonemia determine an upregulation of NMDA glutamate receptors with acute increases intracellular free calcium which, activates nitric oxide synthase (NOS) after binding to calmodulin, leading to an increased production of nitric oxide (NO). NO is able to activate soluble guanylate cyclase, resulting in increased formation of cGMP[90], whereas their downregulation upon prolonged exposure to ammonia leads to impairment of cGMP synthesis that is responsible for intellectual and memory deficits in chronic hepatic encephalopathy patients[54]. Ammonia impairs the glutamate/NO/cGMP pathway, which includes interference with the activation of soluble guanylate cyclase by NO[90]. Reduction of cGMP synthesized in this pathway has been shown to be responsible for cognitive deficits in animals with experimentally induced hyperammonemia or hepatic encephalopathy[91].

In our study, the activity of GA was not affected by single THC administration at any brain region despite the increase in striatal ammonia concentration. This finding is consistent with previous reports showing no

changes in GA activity after the induction of acute and chronic moderate increase in brain ammonia concentrations.[92, 93]

To study the hypothesis that ammonia could originate in the brain after THC administration, and also to mimic the timeline of the clinical trial, we quantified the plasma, liver and brain ammonia concentration at a later time point (30 min). Remarkably, we found a significant increase in striatal ammonia associated with a corresponding increase in plasma ammonia, that was not present at the earliest time points, while liver, PFC and cerebellar ammonia concentrations did not show any significant changes as within the first 5 min.

5.2 Cannabis, ammonia and neuronal activity in the dorsolateral striatum

The striatum as a critical component of the basal ganglia thalamo-cortical circuit, is involved in the mediation of voluntary locomotor activity[94]. In particular, the dorsal part of the striatum, being the main input structure of the basal ganglia, an ensemble of integrative subcortical nuclei enabling the elaboration of complex motor behavior,[95] has been widely studied for its role in mediating locomotor impairment under effect of THC. A previous study, *in vivo* electrophysiological recordings in rats injected with acute THC, showed a significant reduction in neural activity and altered firing patterns in the striatum in parallel with the reduction of spontaneous locomotor activity and treadmill activity.[96]

It is widely accepted that the cannabis-mediated impairment in the locomotor activity occurs through presynaptic inhibition of striatal outputs mediated by GABAergic CB1R terminals of interneurons or collaterals from striatal MSNs.[97, 98] However, blocking the effect of CB1Rs with the neutral CB1R antagonist NESS 0327 successfully reversed the overall locomotor effects of THC, but not THC effect within the first 5 minutes post-injection, suggesting that during this early time window hypolocomotion is not mediated entirely by CB1R activity. We tested the hypothesis that THC may

alter the excitability of striatal neuronal circuits directly through the activation of CB1R and indirectly through increasing ammonia concentration comparing the effect of THC and NH_4Ac on behavior. In line with previous studies,[99, 100] our results showed lower spontaneous locomotor activity after acute administration of NH_4Ac, with a pattern similar to the behavioral effect THC mediated.[100] To correlate the behavioral effect of the two compounds to their ability in modulating the dorsal striatal neuronal activity in awake mice, we designed two pilot experiments using 2 state-of-the-art techniques such as *in vivo* calcium imaging and *in vivo* electrophysiology in awake mice.

For the *in vivo* calcium imaging experiment, we recorded fluctuations in striatal intracellular calcium levels as an indicator of neuronal activity, that allows to directly visualize the effects of THC and ammonia on striatal neurons involved in the decrease in locomotion. Calcium is a universal intracellular signal in eukaryotic cells, and the concentration of free calcium in cytoplasm is typically four orders of magnitude lower than the extracellular concentration and rises substantially after action potential-driven calcium entry. As calcium sensor, we used a fluorescent protein derived from green fluorescent protein (GFP) fused with calmodulin (GCaMP6s). GCaMP6s alters its conformation in response to calcium binding, increasing the fluoresce of GFP. The brightness (changes in fluorescence compared the basal fluorescence) is used as an indicator of calcium levels. Then we use a two-photon microscope to record calcium fluctuations, which are indicative of neuronal action potentials. As we previously discovered for the behavioral effects, our preliminary imaging data showed for the first time that injections of THC 10 mg/kg and NH_4Ac 3 mM/kg cause also similar significative reduction of neuronal activity in the dorsolateral striatum.

Imaging data were further supported from other preliminary results, obtained with *in vivo* electrophysiological recording. In this experiment, we showed a significant reduction of the firing rates of striatal neurons (NH_4Ac: n=5; THC: n=8), compared to baseline and vehicle. Only a previous study on chronic hyperammonemia coupled the decrease in exploratory activity

with a reduced magnitude of electrically-induced long-term potentiation in cortico-striatal synaptic transmission.[100]

Taken together, these new findings indicate the possibility that THC might alter the excitability of striatal neuronal circuits directly through the activation of CB1R and indirectly inducing a transient region-specific increase in ammonia concentration.

6. LIMITATIONS

Our clinical study reports data on a small number of patients, as well as other few limitations intrinsically linked to this kind of studies. Cannabis use and alcohol intake of participants right before the study session were not taken into account and they might have affected the results. Secondly, demographic parameters such as age, gender, body mass index, ethnic backgrounds as well as inter-individual differences in nicotine and cannabis habits that could have affected ammonia concentrations were not considered in our evaluation.

In the animal study, we only quantified plasma, liver and brain ammonia during the first 5 min after the acute administration of THC: studies with more frequent sampling over a longer duration and after chronic administration of THC are warranted to map the details of brain ammonia economy under cannabis use. Furthermore, in the last two experiments we showed only preliminary data from a limited sample of animals: my future work will complete the ongoing experiments to ensure the cohort size needed for statistical purposes.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

Marijuana is the most abused illicit substance and, at the same time, the legalization for its medicinal use is increasing worldwide. Therefore, scientific studies become imperative to better understand and prevent cannabis effects such as alteration of visuospatial abilities and motor coordination, that impair motoric and driving performances.

The results we presented carry remarkable mechanistic and clinical significance for cannabis research. For the first time, plasma ammonia concentration has been shown to increase after marijuana/THC administration in humans and rodents, indicating that ammonia could be generated in the CNS and not peripherally as previously described. Furthermore, we were able to use state-of-art technique to show real time similarities in the effect of ammonia and THC on the striatal neuronal activity, area that showed the significant increase in ammonia after a single administration of THC.

In summary, these results are the first to report effects of THC mediated by ammonia and indicating ammonia as potential target to treat some cannabis-related side effects.

In future, more accurate techniques could be required to trace the ammonia dynamics after controlled cannabis administration. Recent studies have shown differences in marijuana effects between male and female and between cannabis users at different ages,[82,101,102] and further studies are needed to explain the role of ammonia dynamics in these differences. In addition, to perform a direct exposure of mice to cannabis smoke could elucidate the differences between ammonia produced in the brain and the amount absorbed by the lungs after the smoke and vapor inhalation.

Furthermore, a fundamental goal of neuroscience research is to understand how dynamics in neuronal circuits control behavioral outputs. However, using wildtype mice strongly limited our ability to target specific cell subtypes and/or cellular subdomains. Studies on transgenic mice will help to elucidate the effects of THC and ammonia on different

subpopulations of MSNs and interneurons in this and related brain regions.

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