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The acute effect of cannabis on plasma, liver and brain ammonia dynamics, a translational study

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Abstract

Recent reports of ammonia released in high concentrations during cannabis smoking raise concerns about putative neurotoxic effects. Cannabis (54mg) was administered in a double-blind, placebo-controlled design to healthy cannabis users (n=15) either orally, or through smoking (6.9% THC cigarette) or inhalation of heated vaporized cannabis (Volcano®). Serial assay of plasma ammonia concentrations at 0, 2, 4, 6, 8, 10, 15, 30, and 90 minutes from onset of cannabis administration showed significant time [F(8,297)=2.389, P=0.016], and treatment [F(3,297)=6.243, P=0.0004] effects with robust differences between placebo and edible at 30 (p=0.002), and 90 minutes (P=0.007) and between placebo and vaporized (P=0.02) and smoking routes (P=0.01) at 90 minutes. Furthermore, plasma ammonia positively correlated with blood THC concentrations (P=0.03). To test the hypothesis that this delayed increase in plasma ammonia originates from the brain we administered THC (3 and 10mg/kg IP) to mice and measured plasma, liver, and brain ammonia concentrations at 1, 3, 5 and 30 minutes post-injection. Administration of THC to mice did not cause significant change in plasma ammonia concentrations within the first 5 minutes, but significantly reduced striatal glutamine synthetase (GS) activity (p=0.046) and increased striatal ammonia concentration (p=0.016). Furthermore, plasma THC concentration correlated positively with striatal ammonia concentration (p<0.001) and negatively with striatal GS activity (p=0.030). At 30 minute, we found marked increase in striatal ammonia (P<0.0001) associated with significant increase in plasma ammonia (P=0.042) concentration.

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Author contribution

OAA conceived and designed the study. MAH designed the overall clinical study. OAA, MLZ and DTL designed the animal study. OAA, MLZ, and ZL performed all behavioral experimental studies, OAA, MLZ and BA performed molecular experiments, statistical analysis, prepared figures and wrote the manuscript. All authors contributed to reviewing and editing the manuscript.

In conclusion, the results of these studies demonstrate that cannabis intake caused time and route-dependent increases in plasma ammonia concentrations in human cannabis users and in mice, THC reduced brain GS activity and increased brain and plasma ammonia concentrations.

Keywords

Cannabis; Ammonia; Glutamine synthetase; Glutaminase; Striatum

Introduction

Cannabinoids have been shown to be both beneficial and detrimental to neuronal and glial activity and brain plasticity dependent on the dose, route, cannabinoid in question and experimental conditions (Campbell 2001; Chan et al. 1998; Harper et al. 1977; Heath et al. 1980; Landfield et al. 1988; Rocchetti et al. 2013; Scallet et al. 1987). However, the underlying mechanisms of potential cannabis-induced neurotoxicity remain poorly understood (Scallet 1991). Recent reports demonstrate that cannabis smoke and heated cannabis plant contain high concentrations of ammonia (Bloor et al. 2008; Moir et al. 2008) and chronic parenteral administration of cannabis resin to dogs was associated with significant increase in blood ammonia concentration (de Pasquale et al. 1978) while exposing rabbits to hashish smoke every other day for a period of one month resulted in a marked increase in blood ammonia concentration (Ghoneim et al. 1980). Inhaled ammonia (released during smoking) is absorbed quickly through the large surface area of the lungs into the systemic circulation where it diffuses passively through the blood brain barrier (BBB) (Keiding et al. 2006). The brain, with limited capacity to detoxify ammonia through the urea cycle (Ratner et al. 1960), sequesters ammonia into glutamine by astrocytic glutamine synthetase (GS) enzymes (Cooper and Jeitner 2016). Astrocytic glutamine serves as an energy metabolite or gets shuttled to glutamatergic neurons where it is converted into glutamate by glutaminase or shuttled to GABAergic neurons where it produces GABA by glutamic acid decarboxylase (Albrecht et al. 2007). In addition, part of the astrocytic glutamine leaves the brain to the blood in exchange for large neutral amino acids tryptophan, phenylalanine, and tyrosine (James et al. 1978; Jessy et al. 1990; Mans et al. 1982). Under normal physiological conditions brain GS operates at near maximum capacity (Butterworth et al. 1988; Cooper and Plum 1987; James et al. 1978). An increase in systemic blood ammonia could cause a corresponding increase in brain glutamine and brain ammonia concentrations. Increased brain glutamine may increase brain tyrosine and tryptophan concentrations, which could accelerate brain dopamine and serotonin synthesis (Wurtman and Fernstrom 1975). On the contrary, high brain ammonia is neurotoxic through the disruption of mitochondrial energy metabolism (Lai and Cooper 1991), alteration in neuronal firing patterns (Dyannik et al. 2015), activation of glutamatergic signaling (Albrecht et al. 2007; Hermenegildo et al. 1998), and astrocytic swelling (Gorg et al. 2013). In these studies, we aimed to measure plasma ammonia concentrations during and after controlled cannabis administration to cannabis users via different routes. Additionally, we used a translational approach to examine the acute effect of delta-9-tetrahydrocannabinol (THC) on plasma, liver and brain ammonia concentrations and on the activities of GS and GA at different time points.

Experimental Procedures

Human study

The study protocol was approved by the National Institute on Drug Abuse Institutional Review Board and all participants provided written informed consent. An investigational new drug (IND) exemption was obtained from the Food and Drug Administration and the Drug Enforcement Agency approved cannabis administration. Study procedures took place at the NIDA Intramural Research Program during the interval from September 2014 through September 2015 (ClinicalTrials.gov Identifier NCT02177513).

Study design: The study design was double blind, placebo-controlled, randomized, and crossover study with four sessions. In each session, healthy occasional (smoking frequency 2x/month but <3x/week) and frequent cannabis smokers (smoking frequency 5x/week) were asked to consume a placebo or active oral (baked in a brownie) cannabis dose (contains ≈ 54 mg THC) followed by either placebo or active smoked or vaporized cannabis (contains < 0.001mg THC). Only one active dose was administered in each dosing session [either active brownie, and placebo vaporized, or active brownie and placebo cigarette, or placebo brownie and active vaporized, or placebo brownie and active cigarette] or received both placebo doses [placebo brownie and placebo vaporized, or placebo brownie and placebo cigarette]. Oral cannabis doses were prepared per Duncan Hines® Double Fudge cake-like brownie instructions. The contents of an active or placebo cigarette were ground, baked for 30min at 121°C in aluminum foil, and mixed into equal portions of batter in a muffin tin. Following baking, individual doses were stored frozen, but allowed to thaw refrigerated overnight before dosing. Participants consumed the oral and smoked or vaporized dose *ad libitum* over 10min. Randomization was accomplished with the aid of a random number generator. The order for all four sessions was randomly assigned. Additionally, whether the smoked or vaporized placebo is paired with the active oral dose was also randomly rotate between participants. Randomization was generated by computer algorithm operated by NIDA pharmacy.

Study inclusion criteria were: males and females 18 to 50 years of age, consuming cannabis in the past 3 months. A positive urine cannabinoid screen was required for frequent cannabis smokers. Exclusion criteria were: use of cannabis for medical purposes, history of significant adverse events associated with cannabis intoxication, current physical dependence (DSM-IV) on any drug other than cannabis, caffeine, or nicotine, history or presence of any clinically significant illness. Pregnant or nursing females were also excluded and women in child bearing age were required to use an accepted method of contraception.

The primary outcome measures for this study 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. However, in this report, we will only discuss the effect of controlled cannabis administration on plasma ammonia concentrations in a subgroup of subjects (n=15).

Blood sampling for ammonia concentration: Nine samples were collected in each dosing session; at 0, 2, 4, 6, 8, 10, 15, 30, and 90 min post dose. All samples were collected

in pre-cooled heparinized tubes kept in ice water (4°C) during blood collection and centrifuged immediately [3000g (4°C) X 3 min]. Plasma was collected and maintained at 4°C. Immediately following the last collection, specimens were assayed in duplicate using a commercial ammonia colorimetric assay kit (BioVision Inc., Milpitas, CA) according to the manufacturer's instructions. Ammonia concentration was determined at 570nm wavelength with a Sunrise™ microplate absorbance reader (Tecan Group Ltd., Männerdorf, Switzerland). Inter-assay coefficient of variance of in-house quality controls was <10%.

Animal study

Experimental protocols were reviewed and approved by the National Institute on Drug Abuse (NIDA) Institutional Animal Care and Use Committee and the methods were carried out in accordance with the approved guidelines. C57BL/6J mice (22–30g) obtained from The Jackson Laboratories (Bar Harbor, ME), were group housed in cages with ordinary bedding under 12 hour light/ 12 hour dark cycle with lights on at 6:00 AM, temperature was kept around 21 °C, humidity between 40–70% and with free access to standard rodent chow and water.

Three main experimental groups (n=36 each) were used; vehicle control, THC 3mg/kg, THC 10 mg/kg. Each main group had three subgroups (n=12 each) depending on the time of euthanasia at 1, 3, and 5 min post injection. An additional group (n=24) was added to examine the effect of THC on plasma, liver and brain ammonia at 30 min post injection. This 30-min group had also three subgroups (n=8 each); vehicle control, THC 3mg/kg, THC 10 mg/kg.

Natural THC was provided by the NIDA pharmacy and prepared for IP injection by adding THC to TWEEN® 80 (Sigma Aldrich, St. Louis, MO) and 0.9% saline 1:1:18 (v:v:v) (Long et al. 2010). The total volume injected ranged between 0.25 mL and 0.35 mL depending on the THC concentration and the animal weight.

Locomotor activity testing: Each mouse was placed in a plexiglass open field (OF) arena: 27.3cm x 27.3cm x 20.3cm, (Med. Associates Inc., Georgia, VT). Horizontal movements (distance travelled) were recorded with a video-tracking system and analyzed using a VersaMax software (AccuScan Instruments Inc., Columbus, OH) for 5 min before a single IP injection of THC (or vehicle) was administered. The mice were replaced again in the OF arena for a post-dose session (for either 1, 3, 5, or 30 min) before euthanasia.

Euthanasia, blood collection, plasma THC and plasma ammonia assay: Mice were deeply anesthetized in a CO₂ chamber and euthanized by rapid decapitation. 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 used immediately for the colorimetric ammonia assay and another stored at –20° C for future analysis. Immediately following decapitation liver and brain tissues; prefrontal cortex (PFC), striatum (ST), and cerebellum (Cere) were dissected on ice water (4°C) under light microscopy and transported on dry ice to a –80°C freezer until assayed.

Human blood and mouse plasma THC quantification—Plasma Blood and plasma samples were processed according to a previously published method (Scheidweiler et al. 2016). Briefly, 100 μ L of human blood or 20 μ L mouse plasma 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 onto the LC-MS/MS. Linear ranges were 0.5–100 μ g/L. Intra- and inter-day imprecision were 2.4–8.5% and accuracy were 88.9–115%.

Ammonia determination in plasma, liver and brain tissues: Mouse plasma (25 μ L) was deproteinized with an equal volume of 8% perchloric acid and centrifuged at 4000g (4°C) for 5 min. Specimens were neutralized with 2M potassium bicarbonate and re-centrifuged at 4000g (4°C) for 10 min prior to analysis. Following the last collection, specimens were analyzed as mentioned above.

Brain and liver tissues were homogenized in 20 times w/v of ice-cold ammonia kit buffer (BioVision®) and centrifuged at 4000g (4°C) for 5 min. The ammonia concentration was determined using a commercial ammonia colorimetric assay kit (Biovision®).

Brain glutamine synthetase (GS) enzymatic activity: Brain tissues were homogenized in 20 times (w/v) of ice-cold 60 mmol/L imidazole-HCl buffer (pH 7.5) containing 0.1 mmol/L EDTA and spun at 1000g for 10 min at 4°C. Enzymatic activity was assessed as described by Momosaki et al (Momosaki et al. 2015); 10 μ L supernatant was incubated with 90 μ L of a solution containing 60 mmol/L L-glutamine, 15 mmol/L hydroxylamine HCl, 20 mmol/L Na-arsenite, 0.4 mmol/L ADP, 3 mmol/L MnCl₂, and 60 mmol imidazole-HCl buffer (pH 7.5) for 15 min at 37°C. The reaction was stopped by adding 100 μ L of a solution containing 0.05 mol/L trichloroacetic acid, 0.16 mol/L HCl and 1.18 mol/L FeCl₃ and incubated for 5 min at 37°C. The absorbance of glutamyl hydroxamate formed was determined with a Sunrise™ microplate absorbance at 540nm. GS activity is expressed as mmol of γ -glutamyl hydroxamate/min/mg of proteins. Protein content was determined by the Pierce™ BCA Protein Assay Kit (ThermoFisher®, Halethorpe, MD)

Brain glutaminase (GA) enzymatic activity: Brain tissues were homogenized in 20 times (w/v) of ice-cold TES buffer (25 mM Tris-HCl, 0.2 mM EDTA, 0.33 M sucrose, pH 8.0) added with a protease inhibitor cocktail (Roche®). Samples were solubilized with TX-100 at a final concentration of 1% (v/v) and then spun at 100,000g for 30 min at 4°C (Blanco et al. 2015). Protein concentration was measured by the Pierce™ BCA Protein Assay Kit. The enzyme activity was assayed as previously described by Heini et al and Blanco et al (Blanco et al. 2015; Heini et al. 1987) Briefly, 25 μ L supernatant was incubated for 1 h at 37°C with 35 μ L reagent one containing 100 mM potassium phosphate, 171 mM L-glutamine and 1.5 mM NH₄Cl, pH 8.0. The reaction was terminated by adding 10 μ L 10% tricarboxylic acid (TCA). Blanks and reagent one were incubated separately and mixed after the addition of TCA. Samples and blanks were spun at 4,000g for 10 min. A 5 μ L aliquot was add to 150 μ L reagent two containing 0.2 M potassium phosphate, 72 mM mercaptoethanol in ethanol and 186mM o-phthalaldehyde in ethanol, pH was adjusted to

7.4. After incubation (45 min) at room temperature in the dark, the absorbance of the ammonia produced was measured at 405 nm and compared to the absorbance generated by a standard curve of NH_4CL treated with reagent two. GA activity is expressed as mmol ammonia/min/mg of proteins (Blanco et al. 2015).

Statistical analysis—All data are presented as mean $\pm$ SEM (standard error of the mean). Analysis of variance (ANOVA) with time and treatment (THC route) was used to examine the changes in plasma ammonia following cannabis administration in human subjects. The effect of THC (3mg/ kg, 10mg/ kg and vehicle control) on each outcome variable (locomotor activity, plasma and tissue ammonia, and brain GS and GA enzyme activities) was tested using One-way ANOVA and the relationship between plasma THC concentrations each variable and was examined using Pearson correlation. (Prism vs. 4, GraphPad software, La Jolla, California). Results were considered significantly different when $p < 0.05$.

Results

Demographics: The cohort for this pilot study consisted of 15 cannabis users with a mean ($\pm$ SEM) age of 30.5 ± 2.7 and 27.4 ± 2.6 years for frequent ($n=6$) and occasional ($n=9$) cannabis smokers, 6 females, 12 African Americans and 3 Caucasian. Seven subjects reported current nicotine smoking and 14 drank alcohol but none met alcohol abuse or dependence criteria. As detailed in table 1, frequent and occasional users reported starting cannabis use around ages $12.8 (\pm 0.7)$ and $16.4 (\pm 1.2)$ respectively. The cumulative durations of cannabis use were $14 (\pm 2.8)$ and 5.6 ± 1.5 years and the average number of joints/ week were $122.0 (\pm 53.7)$ and $1.7 (\pm 0.3)$ while the peak numbers were $156.0 (\pm 51.6)$ and $16.3 (\pm 7.9)$ joints/ weeks in frequent and occasional users respectively.

The effect of controlled cannabis administration on plasma ammonia concentration in human cannabis users

Plasma ammonia concentrations increased over time in subjects who received active cigarette cannabis compared to placebo (Table 2). Two-way ANOVA revealed a significant effect of treatment [$F(3,297)=6.243$, $P=0.0004$], and time [$F(8, 297)=2.389$, $P=0.016$], but no interaction between the two factors [$F(24, 297)=0.4715$, $P=0.9$]. Posthoc t-test showed significant increase in plasma ammonia at 30 minutes in oral compared to placebo cannabis ($t=3.603$, $df=18$, $P=0.002$) and at 90 minutes in vaporized ($t=2.077$, $df=17$, $P=0.026$), smoked ($t=2.722$, $df=16$, $P=0.015$) and oral ($t=3.043$, $df=16$, $P=0.007$) cannabis compared to placebo (Fig 1A). Furthermore, a significant positive correlation was evident between plasma ammonia and blood THC concentrations ($r=0.1208$, $P=0.037$, Fig 1B).

This pattern of delayed increase in plasma ammonia concentration at 30 and 90 minutes could not be explained by simple diffusion of inhaled ammonia from the lung to systemic circulation. We hypothesized that THC first increases ammonia concentration in the brain which then diffuses back through the BBB to the plasma after reaching high enough concentrations leading to the observed delayed increase in plasma ammonia concentration. To test our hypothesis, we administered THC to mice and measured plasma and brain ammonia concentrations within the first 5 minutes to assay plasma and tissue ammonia

concentrations after single THC administration. At this early time window, we expected to find no increase in plasma or liver ammonia, but early elevation in brain ammonia. We then added a 30 minute group to examine whether plasma ammonia will increase at that delayed time point as we observed in the human study.

Animal study results

Plasma THC concentrations

Administration of two different THC doses; 3 and 10mg/kg resulted in detectable THC levels in the mouse plasma at 1-, 3- and 5-minutes post-injection. Higher plasma THC concentrations were measured at the 5-minute time point following the administration of the 3mg/kg dose. However steady increase in plasma THC concentrations were observed after the 10mg/kg dose (Fig 2). The wide variability and overlap in plasma THC concentrations between the two doses prompted us to analyze the outcome data (locomotor activity, plasma and tissue ammonia, and brain GS and GA enzyme activities) twice; first based on THC dose (control, vs. 3mg/kg vs. 10mg/kg) using a one-way ANOVA, and second based on the relationship between each variable and plasma THC concentration using Pearson correlation.

The effect of THC on locomotor activity

Spontaneous locomotor activity changes from pre to post THC dose showed significant reduction ($F=17.41$, $p<0.0001$, Fig 3A) and robust negative correlation with plasma THC concentration ($r=-0.254$, $p=0.007$, Fig 3B). Similarly, the locomotor activity changes in the 30-min group showed significant reduction after the administration of THC (Supplementary Fig 1A).

The effect of THC on plasma and tissue ammonia concentrations

THC administration was not associated with changes in plasma ammonia concentration ($F=0.679$, $p=0.5$, Fig 4A) and no correlation between plasma THC and plasma ammonia concentrations ($r=-0.085$, $p=0.3$, Fig 4B) within the early time window. However, comparing plasma ammonia concentrations between treatment groups at 30-min showed significant difference [$F(2,21)=3.682$, $P=0.042$] driven mainly by the 3mg/kg THC group [3mg/kg vs. control: $t=2.329$, $df=13$, $P=0.03$, Supplementary Fig 1B].

Similarly, Liver ammonia concentration was not changed by THC ($F=2.308$, $p=0.1$, Fig 4C) and did not show correlation with plasma THC concentration ($r=0.141$, $p=0.19$, Fig 4D) or delayed increase at 30-min (Supplementary Fig 1C). However, a slight but significant increase in striatal ammonia concentration with THC ($F=4.298$, $p=0.016$, Fig 4E) and significant positive correlation ($r=0.321$, $p<0.001$, Fig 4F) between striatal ammonia and plasma THC concentrations were observed during the first 5 minutes and a robust (10-fold) increase in striatal ammonia concentration [$F(2,20)=4.043$, $P=0.033$, Supplementary Fig 1D] induced by both THC doses [3mg/kg vs. control: $t=10.87$, $df=13$, $P<0.0001$ and 10mg vs. control: $t=5.784$, $df=14$, $P<0.0001$] was evident at 30-minutes.

This effect was specific to the striatum since we did not find changes in ammonia concentrations in PFC ($F=1.617$, $p=0.2$, Fig 4G) or cerebellum ($F=0.076$, $p=0.9$, Fig 4I) or correlation between plasma THC and ammonia concentrations in either brain region [PFC ($r=0.116$, $p=0.2$, Fig 4H), and cerebellum ($r=-0.058$, $N=108$, $p=0.5$, Fig 4J)] during the first 5 minutes or at 30-minutes post injection [PFC [$F(2,21)=0.1652$, $P=0.8$, Supplementary Fig 1E], cerebellum [$F(2,21)=1.068$, $P=0.3$, Supplementary Fig 1F]

The effect of THC on GS activity

Since brain ammonia is handled by two main enzymes; astrocytic GS and neuronal GA, we measured striatal GS activity and found significant reduction ($F=3.243$, $p=0.046$) caused by the high THC dose (mean $\pm$ SEM for control vs. THC 10mg/kg = 28.16 ± 1.5 vs. 23.11 ± 1.3 , $p=0.016$, Fig 5A) and negative correlation between plasma THC and striatal GS activity ($r=-0.284$, $p=0.030$, Fig 5B). We did not observe similar effect for THC on GS activities in PFC ($F=0.494$, $p=0.6$, Fig 5C) or cerebellum ($F=0.929$, $p=0.4$, Fig 5E) and there was no correlation between plasma THC and GS activities in these brain regions (PFC: $r=-0.143$, $p=0.2$, Fig 5D, cerebellum: $r=-0.199$, $p=0.13$, Fig 5F).

The effect of THC on GA activity

The reduction in striatal GS activity was not associated with any changes in GA activity in the striatum ($F=0.146$, $p=0.8$, Fig 6A), PFC ($F=0.047$, $p=0.9$, Fig 6C), or cerebellum ($F=1.348$, $p=0.2$, Fig 6E) and no correlation between plasma THC concentration and GA activity in the striatum ($r=0.023$, $p=0.8$, Fig 6B), or PFC ($r=0.024$, $p=0.8$, Fig 6D), was found but we noticed a trend toward positive correlation in the cerebellum ($r=0.259$, $p=0.063$, Fig 6F).

Discussion

To the best of our knowledge, this is the first study that quantifies ammonia concentrations following controlled administration of THC in human cannabis users and in mice. Our human study results provide compelling evidence that cannabis administration causes route and time-dependent increases in plasma ammonia concentrations and this cannabis-induced increase in plasma ammonia correlated positively with blood THC concentration (Fig 1). Using a back translational approach we were able to show a significant-THC plasma concentration dependent-effect of acute THC administration on striatal ammonia concentration associated with reduction in GS activity. Furthermore, we reported a remarkable 10-fold increase in striatal ammonia associated with significant increase in plasma ammonia at 30-minutes post THC administration in mice (Supplementary Fig 1).

Interestingly, the chemical structure of THC ($C_{12}H_{30}O_2$) does not contain ammonia or nitrogen molecules. However, the cannabis plant material releases ammonia during heating or smoking (Bloor et al. 2008; Moir et al. 2008) possibly due to the cleavage of phenylalanine by phenylalanine ammonia lyase enzyme (Hyun et al. 2011).

Inhaled smoked and vaporized heated cannabis plant was associated with increases in plasma ammonia concentrations that reached significance at 90 min (Table 1 and Fig 1). While an increase in ammonia following the oral cannabis administration was observed at

both 30 and 90 min, but not immediately following the consumption of cannabis material. Ammonia released in main stream smoke of NIDA THC cigarette is significantly lower compared to side stream smoke (Bloor et al. 2008). The observed increase in plasma ammonia at 90 min in smoked and vaporized routes could not be explained by the direct inhalation of ammonia. Similarly, the consumption of a brownie containing cannabis resulted in a delayed increase in plasma ammonia concentrations at 30 and 90 min. Following oral cannabis, any ammonia produced through the digestion of cannabis plant material should only cause an increase in the portal circulation ammonia concentrations, which is handled immediately by the hepatic urea cycle without causing an increase in systemic ammonia concentrations. These intriguing findings suggest that the observed delayed increase in plasma ammonia concentration is not attributed to direct effect of ammonia in cannabis plant, but more likely due to an indirect effect of THC on brain ammonia economy.

To examine this hypothesis, we administered THC to mice and measured plasma, liver and brain ammonia concentrations within the first 5 minutes. We used this narrow time window to investigate the source of THC-induced increase in plasma ammonia. Early following controlled cannabis administration in human subjects, plasma ammonia concentration was not significantly elevated (Fig 1). Our animal study results showed that THC administration did not cause increases in plasma (Fig 4A) or liver ammonia concentrations (Fig 4C). However striatal ammonia concentration (Fig 4E) was slightly but significantly elevated. This finding of rapid THC-induced increase in brain ammonia while plasma and liver ammonia concentrations are still within control levels suggests a central source for the observed delayed increase in plasma ammonia in human cannabis users. We then tested the delayed effect of THC at 30 minutes and found robust increase in striatal ammonia associated with corresponding increase in plasma ammonia while liver, PFC and cerebellar ammonia concentrations did not show significant changes. These findings lend more evidence to the hypothesis that the observed THC-induced increase in plasma ammonia originates from the brain.

Furthermore, we measured the activities of the two main ammonia metabolizing enzymes; GS and GA and found significant reduction in striatal GS activity following the administration of THC (Fig. 5A). Moreover, we found significant negative correlation between plasma THC concentration and striatal GS activity (Fig. 5B). These results suggest that THC suppresses striatal GS activity and increases brain ammonia concentration within minutes from acute THC injection while plasma and liver ammonia concentrations remain unchanged.

The reduction in striatal GS activity after single THC dose is consistent with a previous report which demonstrated that THC administered prenatally caused reduction in astrocytic GS expression in mice (Suarez et al. 2002). Similarly, GS activity was reduced in mixed-cell aggregating brain cell cultures after repeated, but not single, THC treatment (2 μ M) for 10 days (Monnet-Tschudi et al. 2008). Interestingly, THC-induced reduction in striatal GS activity was not observed in the cerebellum or PFC which suggests that the effect of THC on GS activity is brain region specific. The underlying mechanism behind GS suppression is not clear. Glutamine accumulation was found to reduce GS activity (Butterworth et al.

1988). Also the reduction in GS activity could stem from a direct effect of THC or indirectly through the reduction of ATP availability since GS is an energy demanding enzyme (Rose et al. 2013) and cannabis reduces brain ATPase enzyme activity (Calderon et al. 2010). Regardless of the mechanism, even slight reductions in GS activity could have a noticeable effect on brain ammonia concentration given that the activity of brain GS is near maximum capacity (Butterworth et al. 1988; Cooper and Plum 1987; James et al. 1978). It is possible that the reduction in striatal GS activity in mice receiving THC does not apply to human cannabis users since the cannabis-induced increase in plasma ammonia could cause corresponding increase in brain ammonia and it has been demonstrated that an increase in brain ammonia elicits an increase in GS activity (Cudalbu et al. 2012). Against this argument that the increase in plasma ammonia took place at a later time point (30–90 minutes) from cannabis intake in humans while the reduction in GS activity in mice was observed earlier while plasma ammonia concentration was not elevated. However, to directly answer this relevant question in human cannabis users, further investigation using ^{13}C spectroscopy is needed to measure, in real-time, the effect of controlled cannabis administration on glutamate-glutamine cycle dynamics (Sailasuta et al. 2010).

Importantly, the suppression of GS activity, could lead to accumulation of glutamate in the astrocytes and reduced capacity of astrocytic glutamate transporter that could result in increased synaptic glutamate concentration, activation of neuronal NMDA receptors and potential neurotoxicity (Lee et al. 2010).

Interestingly, the activity of GA in the striatum or other brain regions (Fig. 6) was not affected by single THC administration 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 concentration (Albrecht et al. 1996; Cooper et al. 1985).

The fast increase in striatal ammonia concentration associated with reduction in GS activity and then delayed increase in ~~but normal~~ plasma ~~and liver~~ ammonia concentrations suggests that the ammonia could originate in the brain. In fact, high brain ammonia concentrations could diffuse back across the BBB to plasma (Keiding et al. 2006; Sorensen 2013). However, the specific effect of THC on BBB integrity and back diffusion of ammonia requires further investigation employing ^{13}N -spectroscopy (Keiding et al. 2006).

The possible relationship between THC-induced increase in striatal ammonia concentration and the reduction in spontaneous locomotor activity remains to be resolved. The clinical relevance of this point cannot be over stated because of the well characterized motoric side effects of cannabis smoking leading to higher risk for motor vehicle crashes during intoxication (Rogeberg and Elvik 2016). The striatum is a critical component of the basal ganglia thalamo-cortical circuit which mediates voluntary locomotor activity (Ameri 1999). Several studies have demonstrated that acute injection of ammonia is associated with transient dose-dependent reduction in locomotor activity (Giguere and Butterworth 1984; Grzeda and Wisniewska 2009; Hindfelt and Siesjo 1971). However, in order to investigate a potential link between the striatal ammonia concentration and locomotor activity, further

studies employing microdialysis techniques and specific ammonia-chelating agents are needed.

Our results should be viewed in the light of several limitations. The pilot nature of the human study did not permit analyzing the data based on cannabis use frequency. In addition, we did not measure THC concentration in the air of vaporized cannabis. However, Gieringer et al (2004) measured THC concentration in three samples of vaporized cannabis and reported that on average, the recovered THC amounted to 1.95% of the original weight of the sample, or 47% of the original THC in the crude sample (Gieringer D et al. 2004). Furthermore, in the animal study we quantified plasma, liver and brain ammonia during the first 5 min of acute administration of THC and during only one delayed (30-min) time point. Studies adopting other brain regions, more frequent sampling over longer duration and chronic administration of THC and cannabis are warranted to map the details of brain ammonia economy under cannabis use. Finally, the methodology for measuring plasma and brain ammonia requires specific attention. We performed all mice experiments at the beginning of dark phase (starting at 18:00 hr) to minimize the circadian variability in plasma ammonia concentration (Buchmann et al. 1996). We used a traditional colorimetric method according to the manufacturer's protocol. We followed strict specimen integrity guidelines by maintaining specimens at 4°C and excluding any hemolyzed specimens from analysis. Blanks, calibrators, in-house quality controls and specimens were analyzed in duplicate, and absorbances averaged. For liver and brain tissues, we also maintained collection/storage temperatures and ran all tissue samples under the same conditions, at the same time, to minimize variability and allow valid comparisons. Because of these precautions, our plasma ammonia values are consistent with previously reported concentrations (Buchmann et al. 1996; Jessy et al. 1990; Sarna et al. 1979) and the variability in ammonia concentration in different brain regions is also consistent with the literature (Buchmann et al. 1996; Del Rosso et al. 2016; Keiding et al. 2006; Swamy et al. 2005).

Despite these experimental limitations our results carry remarkable mechanistic and clinical significance for cannabis research. For the first time, we demonstrate that plasma ammonia concentration increases after controlled cannabis administration and we provide evidence that this peripherally detected ammonia could be generated centrally in the brain through suppression of striatal GS activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Change in plasma ammonia concentration over time after controlled cannabis administration by different routes in human cannabis users.

A. The administration of cannabis showed significant effect of treatment: $F(3,297)=6.243$, $P=0.0004$, and time: $F(8, 297)=2.389$, $P=0.016$, by two way ANOVA. (*) significant difference between oral and placebo cannabis ($t=3.603$, $df=18$, $P=0.002$) and (**) between vaporized ($t=2.077$, $df=17$, $P=0.026$), smoked ($t=2.722$, $df=16$, $P=0.015$) and oral ($t=3.043$, $df=16$, $P=0.007$) cannabis compared to placebo by two-tailed t-test.

B. The correlation between plasma ammonia and blood THC concentrations.

Figure 2: Mouse plasma THC concentration

Mean ($\pm$ SEM) plasma THC concentration ($\mu\text{g/L}$) at 1, 3 and 5 minutes following IP administration of THC [3mg/kg (1 min: 36.6 ± 6.8 , 3 min: 41.4 ± 3.1 , 5 min: 121.3 ± 7.6) 10mg/kg (1 min: 193.7 ± 15.6 , 3 min: 289.7 ± 27.9 , 5 min: 319.2 ± 19.6)].

Figure 3: Spontaneous locomotor activity (distance travelled) changes () before and after THC administration

A. Significant reduction in locomotor activity [(F=17.41, $p < 0.0001$) by one-way ANOVA] between controls and 3mg/kg [mean difference and 95% CI = 58.0, 18.2–97.8, $p < 0.001$] and 10mg/kg doses [mean difference and 95% CI = 90.49, 56.0–125.0, $p < 0.0001$] by Dunnett's multiple comparisons test.

B. Significant negative correlation between plasma THC concentrations and locomotor activity [Pearson $r = -0.254$, $P = 0.009$].

Figure 4: Plasma, liver and brain ammonia concentration

Comparison between ammonia concentrations after IP administration of vehicle control, 3mg/kg and 10mg/kg THC in (A) plasma ($F=0.679$, $p=0.5$), (C) liver ($F=2.308$, $P=0.3$), (E) striatum ($F=4.298$, $P=0.016$) [vehicle control vs. 3mg/kg: mean difference between and 95% CI = 0.0313, -0.1262 to 0.06362, $p=0.1$ and vehicle control vs. 10mg/kg: -0.1206 and -0.2162 to -0.02503, $P=0.01$ by Dunnett's multiple comparisons test], (G) PFC ($F=1.617$, $P=0.2$), and (I) cerebellum ($F=0.076$, $P=0.9$).

Correlation between plasma THC and ammonia concentrations in (B) plasma ($r=-0.085$, $P=0.3$), (D) liver ($r=0.141$, $P=0.19$), (F) striatum ($r=0.32$, $P<0.001$), (H) PFC ($r=0.116$, $P=0.2$), and (J) cerebellum ($r=-0.058$, $P=0.5$).

Figure 5: Brain glutamine synthetase (GS) enzyme activity

Comparison between GS enzyme activities after IP administration of vehicle control, 3mg/kg and 10mg/kg THC in (A) the striatum ($F=3.243, P=0.046$), [vehicle control vs. 3mg/kg: mean difference between and 95% CI = 3.20, -0.1296 to 7.696 , $p=ns$ and vehicle control vs. 10mg/kg: 5.052 and 0.5006 to 9.604 , $P=0.04$ by Dunnett's multiple comparisons test], (C) PFC ($F=0.494, P=0.6$), and (E) cerebellum ($F=0.929, P=0.4$).

Correlation between plasma THC and GS activity in (B) striatum ($r=-0.284, P=0.03$), (D) PFC ($r=-0.143, P=0.2$), and (F) cerebellum ($r=-0.199, P=0.13$).

Figure 6: Brain glutaminase (GA) enzyme activity

Comparison between GA enzyme activities after IP administration of vehicle control, 3mg/kg and 10mg/kg THC in (A) the striatum ($F=0.146, p=0.8$), (C) PFC ($F=0.047, p=0.9$), and (E) cerebellum ($F=1.348, p=0.2$).

Correlation between plasma THC and GA activity in (B) striatum ($r=0.023, P=0.8$), (D) PFC ($r=0.024, P=0.8$), and (F) cerebellum ($r=0.259, P=0.06$).

Table 1:

Subject demographics: M=Male, F=Female, AA=African American, C= Caucasian, Y=year

ID	Age(Y)	Sex	Race	Cannabis use				
				Age of onset (Y)	Duration (Y)	Use pattern	Avg Joint/Wk	Peak Joint/Wk
G	37	M	AA	12	15	Daily	378	378
H	29	F	AA	11	17	Daily	105	147
I	24	M	AA	17	1	Weekly	2	3
L	38	M	AA	16	22	Daily	126–154	126–154
O	33	M	AA	14	19	Daily	15	35
P	45	F	AA	26	12	Weekly	4	21
Q	21	F	C	16	1	Weekly	1	0.5–1
R	33	M	AA	16	8	Weekly	0.5–1	72–105
S	21	M	AA	11	4	Daily	63	210
T	25	M	AA	13	7	Daily	45	45
U	22	F	AA	16	1	Weekly	2	4
V	28	F	AA	16	5	Weekly	2	8
W	22	F	C	14	4	Weekly	1	1
X	21	M	C	12	5	Weekly	0.5–1	2.5–5
Y	31	M	AA	15	14	Weekly	3	35
Frequent users Mean ± SEM	30.5±2.7			12.8±0.7	14.0±2.8		122.0±53.7	156.0±51.6
Occasional users Mean ± SEM	27.4±2.6			16.4±1.2	5.6±1.5		1.7±0.3	16.3±7.9