



Metabolomics and integrated network analysis reveal roles of endocannabinoids and large neutral amino acid balance in the ayahuasca experience

Francisco Madrid-Gambin^{a,b,*}, Alex Gomez-Gomez^a, Arnau Busquets-Garcia^c, Noemí Haro^a, Santiago Marco^{b,h}, Natasha L. Mason^d, Johannes T. Reckweg^d, Pablo Mallarón^d, Lilian Kloft^d, Kim van Oorsouw^e, Stefan W. Toennes^f, Rafael de la Torre^g, Johannes G. Ramaekers^d, Oscar J. Pozo^{a,**}

^a Applied Metabolomics Research Group, IMIM-Institut Hospital del Mar d'Investigacions Mèdiques, 08003 Barcelona, Spain

^b Signal and Information Processing for Sensing Systems, Institute for Bioengineering of Catalonia (IBEC), The Barcelona Institute of Science and Technology, 08028 Barcelona, Spain

^c Cell-type mechanisms in normal and pathological behavior Research Group, IMIM-Institut Hospital del Mar d'Investigacions Mèdiques, 08003 Barcelona, Spain

^d Department of Neuropsychology and Psychopharmacology, Faculty of Psychology and Neuroscience, Maastricht University, 6200 MD Maastricht, The Netherlands

^e Department of Clinical Psychological Science, Faculty of Psychology and Neuroscience, Maastricht University, 6200 MD Maastricht, The Netherlands

^f Institute of Legal Medicine, University Hospital, Goethe University, Frankfurt/Main, Germany

^g Integrative Pharmacology & Systems Neuroscience Group, IMIM-Institut Hospital del Mar d'Investigacions Mèdiques, 08003 Barcelona, Spain

^h Department of Electronics and Biomedical Engineering, Universitat de Barcelona, Barcelona 08028, Spain

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ABSTRACT

There has been a renewed interest in the potential use of psychedelics for the treatment of psychiatric conditions. Nevertheless, little is known about the mechanism of action and molecular pathways influenced by ayahuasca use in humans. Therefore, for the first time, our study aims to investigate the human metabolomics signature after consumption of a psychedelic, ayahuasca, and its connection with both the psychedelic-induced subjective effects and the plasma concentrations of ayahuasca alkaloids.

Plasma samples of 23 individuals were collected both before and after ayahuasca consumption. Samples were analysed through targeted metabolomics and further integrated with subjective ratings of the ayahuasca experience (i.e., using the 5-Dimension Altered States of Consciousness Rating Scale [ASC]), and plasma ayahuasca-alkaloids using integrated network analysis. Metabolic pathways enrichment analysis using diffusion algorithms for specific KEGG modules was performed on the metabolic output.

Compared to baseline, the consumption of ayahuasca increased N-acyl-ethanolamine endocannabinoids, decreased 2-acyl-glycerol endocannabinoids, and altered several large-neutral amino acids (LNAAs). Integrated network results indicated that most of the LNAAs were inversely associated with 9 out of the 11 subscales of the ASC, except for tryptophan which was positively associated. Several endocannabinoids and hexosylceramides were directly associated with the ayahuasca alkaloids. Enrichment analysis confirmed dysregulation in several pathways involved in neurotransmission such as serotonin and dopamine synthesis.

In conclusion, a crosstalk between the circulating LNAAs and the subjective effects is suggested, which is independent of the alkaloid concentrations and provides insights into the specific metabolic fingerprint and mechanism of action underlying ayahuasca experiences.

Abbreviations: , 2-AG, 2-arachidonoyl glycerol; , ASC, 5-Dimension Altered States of Consciousness Rating Scale; 5HIAA, 5-hydroxyindoleacetic acid; , AAA, aromatic amino acid; , AEA, anandamide; , BCAA, branched-chain amino acid; , DGLEA, dihomogamma-linolenylethanolamide; , FDR, false discovery rate; , KEGG, Kyoto Encyclopedia of Genes and Genomes; , LNAA, large-neutral amino acid; , LC-MS/MS, liquid chromatography–tandem mass spectrometry; , LLOQ, lower limits of quantitation; , LPC, lysophosphatidylcholine; , mPLS-DA, multilevel-partial least squares discriminant analysis; N, N-dimethyltryptamine, DMT; , rCCA, Regularized canonical correlation analyses; , VAS, visual analogue scale.

* Corresponding author at: Applied Metabolomics Research Group, IMIM-Institut Hospital del Mar d'Investigacions Mèdiques, 08003 Barcelona, Spain.

** Corresponding author.

E-mail addresses: fmadrid@imim.es, francisco.madrid.gambin@gmail.com (F. Madrid-Gambin), opozo@imim.es (O.J. Pozo).

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1. Introduction

Ayahuasca is a psychedelic brew originally from South America used for healing and religious purposes by Amazonian indigenous cultures [60,64]. Nowadays, the consumption of ayahuasca is part of syncretic ceremonies of modern Brazilian religions, including Santo Daime and União do Vegetal [34]; however, its consumption has also rapidly spread to the United States and Europe [19,56].

Ayahuasca is traditionally made with the bark of *Banisteriopsis caapi* (*B. caapi*) and the leaves of *Psychotria viridis* (*P. viridis*). These plant species provide a synergistic combination of alkaloids responsible for the strong psychedelic effects, such as β -carboline alkaloids (i.e., harmine, tetrahydroharmine, and harmaline) and N,N-dimethyltryptamine (DMT), that are extracted from the plant materials during the brew preparation [42]. While the β -carbolines present in *B. caapi* have been found to inhibit monoamine oxidase (MAO), and thus, are required to avoid the metabolism of DMT by visceral MAO [44], DMT from *P. viridis* provides intense alterations in sensory integration and conscious awareness experiences [57].

Although there is a lack of biochemical knowledge on the human consumption of ayahuasca, it has a dual effect on human health. The arise of clinical research on ayahuasca provides early evidence for treatment efficacy and safety for a range of psychiatric conditions [1,23,62,78]. However, it also involves risks. The blockage of MAO implies the accumulation of serotonin at the nerve terminals, which in combination with other serotonergic drugs potentially leads to serotonin syndrome [4,18,38]. There is also a risk of developing psychosis or nonpsychotic mania in vulnerable users or those with a family history (Rafael G [13]).

The understanding of biological processes underlying the ayahuasca-induced subjective effects may dispose of potential evidence for therapeutic use [53]. However, the mechanism and molecular pathways involved in the impact of ayahuasca on healthy humans are yet to be investigated. There is knowledge that a psychedelic/ayahuasca experience is triggered by 5HT_{2A} receptor stimulation [45,68]. Nevertheless, little is known about the cascade of neurochemical alterations that are triggered by 5-HT_{2A} receptor stimulation downstream the neural pathways. The study of the metabolome may provide significant knowledge about which pathways ayahuasca may alter. The use of comprehensive metabolomics combines advanced analytical chemistry techniques with sophisticated data analyses to enable the high-throughput profiling of small molecule metabolites in system biology [80]. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) and nuclear magnetic resonance are popular techniques to explore alterations in the metabolome [25]. Indeed, the use of these techniques to investigate dysregulated metabolic pathways has yielded important insights into biological/physiological processes improving our understanding of both physiology and pathology [80].

While several metabolomic studies have been carried out on the ayahuasca beverage [22,31,66], to date, scarce metabolomics investigations have been conducted in humans [75]. Previous studies in other drugs with psychedelic effects using animal models provided support on alteration of amino acids [79], lipids (M. [82]), steroids [5], and energy metabolism [79]. In this sense, it has been suggested that activation of 5-HT_{2A} also modulates endocannabinoids (Rafael G. [12]; Rafael Guimarães [14,49]). Moreover, Riba and colleagues found increased normetanephrine excretion, a metabolite of norepinephrine, after ayahuasca consumption [58]. These findings provided intriguing support for the view that the peripheral metabolome may be associated with brain metabolic alterations after the consumption of ayahuasca.

Nowadays, due to its potential therapeutic value in treating increasing psychiatric illness, there is an urgency to decipher the pharmacological mechanism of ayahuasca. In the current investigation, we first applied a targeted LC-MS/MS metabolomics approach to discover the plasma metabolomic signature produced by ayahuasca consumption. Then, an integrative network was used to identify the metabolic changes associated with the ayahuasca alkaloids. We also evaluated the

connection of the ayahuasca-subjective effects with plasma metabolites to help in the elucidation of its mechanism of action. Lastly, pathways enrichment analysis mapped relevant metabolites to pathways, reactions, or relation networks elucidating altered pathways.

2. Materials and methods

2.1. Subjects and study design

Twenty-three healthy volunteers (14 male, 9 female) with an age of 37.9 ± 10.3 years (mean \pm SD) were enrolled in a within-subject observational study. Participants were members of the Dutch chapter of the Santo Daime church [7]. This is a religious tradition originating from Brazil that has spread globally. Members take part in ceremonies consisting of *trabalhos* (Portuguese for ‘works’), lasting several hours in which singing and dancing are alternated with periods of silence. Ayahuasca is drunk as part of the ceremony. Exclusion criteria were ferromagnetic devices/implants (MRI contraindications), pregnancy and use of (medicinal) substances in the past 24 h. Participants were assessed on consecutive testing days: on the first test day, the participants arrived at the testing facility at Maastricht University the day before the ceremony (hereafter referred to as baseline). The second test day took place the next day when all participants attended an ayahuasca ceremony immediately prior to arriving at the test facility under the influence of ayahuasca. In order to standardize the testing time for each participant, the Santo Daime members consumed ayahuasca individually, separated by a one-hour time window. The ayahuasca ceremony was organized and supervised by the Santo Daime church. The research team was not involved in the organization of the ceremonies or the production, dosing, and administration of ayahuasca.

The study was conducted according to the Declaration of Helsinki (1964) and amended in Fortaleza (Brazil, October 2013) and in accordance with the Medical Research Involving Human Subjects Act (WMO) and was approved by the Academic Hospital and University’s Medical Ethics committee (NL70901.068.19/METC19.050). All participants were fully informed of all procedures, possible adverse reactions, legal rights and responsibilities, expected benefits, and their right to voluntary termination without consequences.

2.2. Psychological measures

The psychological effects elicited by ayahuasca were measured by means of the validated 5-Dimension Altered States of Consciousness Rating Scale (ASC) at the end of the second test day. ASC is a 94-item self-report scale that assesses the alteration of the participants from conventional waking consciousness, with a Cronbach’s alpha range between 0.88 and 0.95 [10,11,71]. Participants were instructed to indicate to what extent each statement applied to their own experience by putting a mark on a vertical 10-cm visual analogue scale (VAS) with anchor ends representing the two extremes of the experience: 0% indicating “No, not more than usually”; and 100% “Yes, much more than usually”. The ASC assesses 11 subscales consisting of (I) the experience of unity, (II) spiritual experience, (III) blissful state, and (IV) insightfulness; (V) disembodiment, (VI) impaired control and cognition, and (VII) anxiety; (VIII) complex imagery, (IX) elemental imagery, (X) audio-visual synaesthesia, and (XI) changed meaning of percepts.

2.3. Ayahuasca brew alkaloids

The alkaloid concentrations of the ayahuasca brew used in the ceremony were determined as in previous studies [33] after dilution and extraction (1-chlorobutane/ether; 1:1, v/v), utilizing high-performance LC–electrospray ionization–MS. Pure reference substances of DMT (Cerilliant, Round Rock TX, US), harmine and harmaline (Aldrich Chemistry, St. Louis MO, US) and of tetrahydroharmine (THH; LGC GmbH, Luckenwalde, Germany) were used for calibration.

2.4. Plasma alkaloid concentrations

Blood samples collected at baseline and 90 min after drinking ayahuasca were aliquoted, encoded, and frozen at -80°C until they were used. Analysis of the alkaloids DMT, harmine, harmaline and tetrahydroharmine in serum samples (200 μl) was performed after extraction with ethyl acetate using LC-MS/MS (Agilent, Waldbronn, Germany). Calibration curves covered the range 0.25 – 40 ng/ml with lower limits of quantitation (LLOQ) of DMT 0.077, harmine 0.13, harmaline 0.23, and tetrahydroharmine 0,18 ng/ml.

2.5. Metabolomics analysis

After protein precipitation, blood samples were analysed via six protocols, which assessed each family of compounds using previously reported methods [20,21,39,40,47,50]. A set of 164 targeted biomarkers, composed of 12 compounds related to endocannabinoids, 42 markers related to amino acid metabolism, 13 steroids, 25 markers related to energy metabolism, 26 acylglycerols, 24 ceramides, 18 lysophosphatidylcholines (LPCs) and, 4 compounds of choline metabolism; was determined by selected reaction monitoring by LC-MS/MS system consisting of an Acquity UPLC instrument (Waters Associates, Milford, MA, USA) coupled to a triple quadrupole (TQS Micro, Waters) mass spectrometer. MassLynx software V4.1 (Waters Associates) was used for peak integration and data management.

2.6. Statistical analysis

To describe the characteristics of the participants, data were expressed as the mean and standard deviation, proportion, or N (%). The datasets were imported to R software version 4.0.3 for statistical analyses (R [55]). Metabolites with more than 80% of samples below the limit of detection or limit of quantification were removed from the final dataset. Before performing a principal component analysis to detect the presence of outliers, missing values were replaced by half of the minimum value within the dataset. Shapiro-Wilk test was used to assess normality. To evaluate potential pre-post consumption differences concerning the studied metabolites, paired two-tailed Wilcoxon tests were used based on the distribution of the variables. The Benjamini-Hochberg procedure was carried out on all analyses to control the false discovery rate (FDR) [3]. An FDR-corrected p-value of < 0.05 was considered statistically significant.

Predictive modelling of the metabolic impact of ayahuasca consumption was conducted by multilevel-partial least squares discriminant analysis (mPLS-DA) on paired samples using the MUVR R package [65]. The generation of the mPLS-DA model was carried out in a repeated double cross-validation [17] framework including a recursive ranking based on Variable Importance in Projection and sequential backward feature elimination and sequential backward feature. The entire operation was repeated twenty times for improved coverage of inner and outer segments and modelling performance. Additionally, the significance of each of the models was assessed by means of a permutation test of 200 iterations between permuted models, with a random assignation of the classes, and the actual model obtained. A p-value lower than 0.05 was considered significant.

2.7. The integrated pathway network construction

Regularized canonical correlation analyses (rCCA) [35,67] were performed on all individuals using the regularization of the empirical covariance matrices. This model serves as an integrative multivariate approach to assess correlations between metabolomics, ayahuasca alkaloids, and the ayahuasca-induced subjective effects. We also included behavioural variables in the consumption, such as the number of days since the last consumption of ayahuasca, the number of accumulated ceremonies, and years in Santo Daime. Lambdas were estimated

utilizing the Shrinkage method [63]. Next, integrative pathway network and the corresponding clustered heat map were built from the rCCA outputs. Clustered heat map dendrograms were computed from hierarchical cluster analysis, using the Euclidean distance and the complete linkage method (Z. [84]). The network graph describes connections between the three datasets based on a similarity score ≥ 0.3 [24].

2.8. Enrichment analysis

Significant metabolites were mapped to metabolic pathways enrichment using the FELLA package [52]. This package provides null diffusion-based enrichment for pathways and specific “Kyoto Encyclopedia of Genes and Genomes” (KEGG) modules in which entities like metabolites, enzymes, and related reactions can suggest insights of pathways overlap and crosstalk, specifically for a studied condition [51]. The package used available KEGG data to build a *knowledge graph* using *Homo sapiens* (T01001, Release 100.0 +/11–11, Nov 21), which included compounds, pathways, and reactions. Then, significant metabolites associated with ayahuasca consumption were used as input and separately mapped within the created structure, generating an enriched graph. The diffusion method (undirected heat diffusion model) of the propagation algorithm was next employed to score graph nodes. For statistical normalization, the parametric z-score was performed using normality approximations. The created *graphml* file was imported into Cytoscape [69] for the network visualization.

3. Results

The characteristics of the individuals regarding demographic, epidemiological, and lifestyle variables are presented in Table 1. The brew contained 0.14 mg/ml of DMT, 4.50 mg/ml of harmine, 0.51 mg/ml of harmaline, and 2.10 mg/ml of tetrahydroharmine. Plasma samples of the twenty-three individuals who participated in the ayahuasca ceremony were analysed for the metabolomics set and the ayahuasca-alkaloids set.

3.1. Metabolic changes after ayahuasca consumption

The mPLS-DA resulted in a model with a classification rate of 100.0%, with a 95% CI of 85.2 and 100.0 (supplemental Fig. 1) with a permutation test p-value of < 0.01 . Thirty-one differential metabolites were extracted from the multivariate modelling, while the Wilcoxon test detected differences for 20 metabolites (FDR-corrected p-value < 0.05). Results from the uni- and multivariate analyses are presented in Table 2.

Overall, most of the metabolites associated with the intake of ayahuasca were involved in either endocannabinoid or amino acid metabolism. After ayahuasca intake, most of the N-acyl-ethanolamine endocannabinoids (e.g. anandamide [AEA], docosahexaenylethanolamide, oleylethanolamide, palmitoylethanolamide, dihomo- γ -linolenylethanolamide [DGLA]) increased in plasma, while the 2-acyl-glycerols (e.g. 2-arachidonoyl glycerol [2-AG], 2-oleoylglycerol, 2-linoleoyl glycerol) decreased. Likewise, a few

Table 1

Clinical and demographic characteristics of the participants of the study.

Variable	Mean \pm SD and N (%)
Sex (male)	14 (60.8%)
Age (years)	37.91 \pm 10.31
Weight (kg)	75.24 \pm 12.10
Height (m)	1.78 \pm 0.06
BMI (kg/m ²)	23.57 \pm 3.32
Years in Santo Daime	14.43 \pm 8.39
Number of ceremonies	566.73 \pm 665.17
Dose (ml)	24.30 \pm 8.32
Dose/weight (ml/kg)	0.33 \pm 0.11

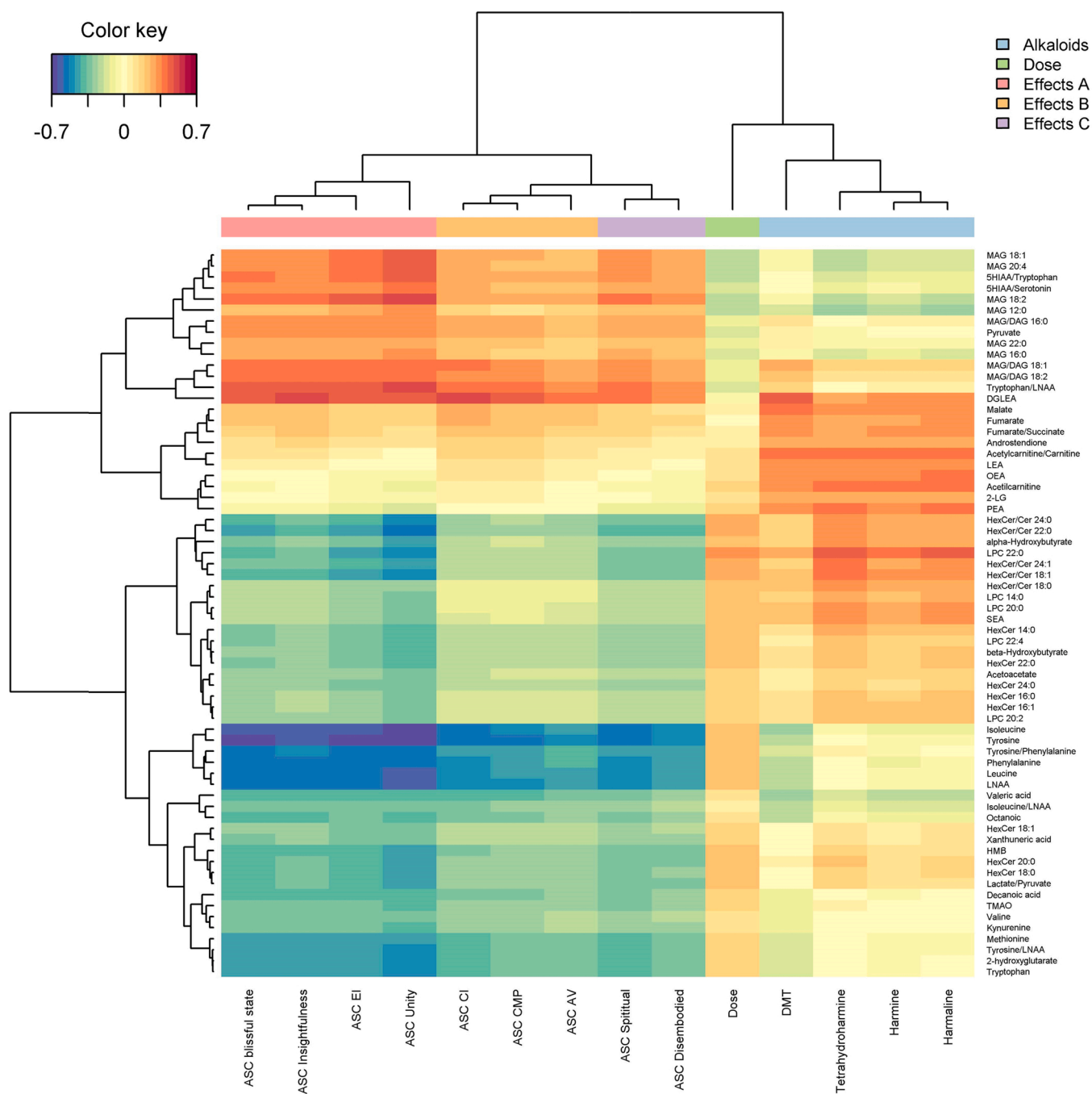


Fig. 1. Heatmap analysis performed by using regularized canonical correlations analysis showing the relation between ayahuasca alkaloids and ayahuasca-subjective effects against metabolomic dataset, after the intake of ayahuasca. Correlation strengths are indicated by the colour key. Abbreviations: 2-AG, 2-arachidonoyl glycerol; 2-LG, 2-linoleoyl glycerol; 2-OG, 2-oleoylglycerol; 5HIAA, 5-hydroxyindoleacetic acid; AEA, anandamide; AV, audio-visual synaesthesia; Cer, ceramide; CI, Complex Imagery; CMP, Changed meaning of perception; DAG, diacylglycerol; DEA, docosatetraenoyl ethanolamide; DGLEA dihomog- γ -linolenoyl ethanolamide; DHEA, docosahexaenoyl ethanolamide; DMT, N,N-dimethyltryptamine; EI, Elemental Imagery; FDR, False Discovery Rate; hexosylceramides; HexCer; MAG, monoacylglycerol; LNAA, large neutral amino acids; LPC, lysophosphatidylcholine; OEA, oleoyl ethanolamide; POEA, palmitoleyl ethanolamide.

monoacylglycerol species and the circulating levels of serotonin pathway metabolites such as 5-hydroxyindoleacetic acid (5HIAA), ratio 5HIAA/tryptophan, and ratio 5HIAA/serotonin also decreased. Concerning the amino acid metabolism, while there was a reduction in the levels of certain large neutral amino acids (LNAAs) such as tyrosine and related ratios (e.g. tyrosine/phenylalanine and tyrosine/LNAA), a rise of leucine/LNAA, isoleucine/LNAA, glutamate, glutamine, and α -hydroxybutyrate were found. Lastly, the concentration of certain steroid metabolites, such as cortisone, 20 α -DHE and 20 β -DHE, increased after ayahuasca intake.

3.2. Integrated pathway network analysis

The dendrogram from the rCCA highlighted five distinguishable clusters that were representative of the ayahuasca dose, ayahuasca alkaloid concentrations (DMT, harmine, tetrahydroharmine, and harmaline), and three clusters of subjective ratings of consciousness (termed as “effects A”, “effects B” and “effects C”), shown in Fig. 1. The rCCA revealed that all subscales except “impaired control and cognition” and “anxiety” of the ASC strongly correlated with clusters of metabolic markers, achieving a similarity score of up to |0.7|. Interestingly, some

Table 2
Differential metabolic signature after ayahuasca consumption (values in response).

Metabolic marker ^a	Description	Baseline	After ayahuasca	P value ^b	FDR	LR ^c
Tyrosine/LNAA	AA metabolism	0.231 (0.202–0.245)	0.193 (0.175–0.22)	< 0.001	0.003	1
DHEA	Endocannabinoid	0.026 (0.019–0.034)	0.04 (0.031–0.062)	< 0.001	0.003	4
OEA	Endocannabinoid	0.080 (0.074–0.106)	0.132 (0.109–0.165)	< 0.001	0.003	2
POEA	Endocannabinoid	0.0048 (0.0036–0.0078)	0.0091 (0.0066–0.0131)	< 0.001	0.003	5
AEA	Endocannabinoid	0.016 (0.014–0.025)	0.034 (0.027–0.04)	< 0.001	0.003	3
5HIAA/Tryptophan	AA metabolism	0.0015 (0.0011–0.0030)	0.0012 (0.0009–0.0016)	< 0.001	0.009	20
5HIAA/Serotonin	AA metabolism	0.217 (0.119–1.275)	0.093 (0.075–0.186)	< 0.001	0.009	28
2-OG	Endocannabinoid	0.171 (0.139–0.212)	0.1 (0.092–0.131)	0.001	0.010	7
5HIAA	AA metabolism	0.066 (0.055–0.12)	0.05 (0.035–0.065)	0.001	0.010	16
DGLEA	Endocannabinoid	0.0072 (0.0061–0.0076)	0.0106 (0.0094–0.0129)	0.001	0.017	6
20 α -DHE	Steroid	0.027 (0.022–0.03)	0.045 (0.031–0.05)	0.001	0.018	8
20 β -DHE	Steroid	0.0073 (0.0060–0.0081)	0.0103 (0.0083–0.0115)	0.001	0.018	18
Glutarate	Energy metabolism	0.063 (0.058–0.077)	0.056 (0.050–0.066)	0.002	0.028	12
Tyrosine	AA metabolism	47.46 (37.94–56.15)	34.74 (27.86–42.80)	0.003	0.028	9
α -Hydroxybutyrate	AA metabolism	0.650 (0.455–0.839)	1.307 (0.994–1.495)	0.003	0.028	10
Leucine/LNAA	AA metabolism	0.144 (0.131–0.163)	0.168 (0.156–0.179)	0.003	0.028	11
2-LG	Endocannabinoid	0.050 (0.033–0.067)	0.027 (0.023–0.036)	0.004	0.039	23
Cortisone	Steroid	0.204 (0.171–0.244)	0.286 (0.235–0.351)	0.005	0.047	14
Tyrosine/Phenylalanine	AA metabolism	1.912 (1.562–2.132)	1.465 (1.282–1.752)	0.005	0.047	15
LEA	Endocannabinoid	0.050 (0.036–0.056)	0.061 (0.050–0.077)	0.006	0.049	19
β -Hydroxybutyrate	Energy metabolism	2.073 (1.515–3.414)	3.123 (1.934–6.411)	0.010	0.079	21
Acetylcarnitine	AA metabolism	4.384 (3.272–5.776)	6.353 (4.064–8.741)	0.012	0.087	24
2-AG	Endocannabinoid	0.020 (0.012–0.024)	0.012 (0.011–0.015)	0.013	0.087	17
Acetylcarnitine/Carnitine	AA metabolism	1.061 (0.882–1.358)	1.194 (0.941–2.171)	0.013	0.087	25
Glutamine/Glutamate	AA metabolism	6.495 (4.422–11.087)	5.093 (3.725–6.528)	0.013	0.087	22
Glutamate	AA metabolism	4.423 (2.807–8.594)	8.510 (6.527–12.374)	0.014	0.087	13
Octanoic	Energy metabolism	0.053 (0.034–0.074)	0.025 (0.02–0.047)	0.014	0.087	29
Glyoxylate	Energy metabolism	1.568 (1.362–2.019)	3.127 (2.473–3.883)	0.016	0.091	26
Isoleucine/LNAA	AA metabolism	0.062 (0.058–0.067)	0.069 (0.063–0.074)	0.025	0.126	30
MAG/DAG 18:1	Acyl glycerol	0.080 (0.047–0.093)	0.054 (0.040–0.060)	0.030	0.139	27
DEA	Endocannabinoid	0.0085 (0.0079–0.0120)	0.0124 (0.0104–0.0140)	0.040	0.164	31

^a Values are medians (IQR).

^b P-values from the two-tailed paired Wilcoxon test.

^c Loading rank of double cross-validated multilevel-PLS-DA. Only significant auto-selected variables are numbered. Abbreviations: 2-AG, 2-arachidonoyl glycerol; 2-LG, 2-linoleoyl glycerol; 2-OG, 2-oleoyl glycerol; 5HIAA, 5-hydroxyindoleacetic acid; AEA, anandamide; DAG, diacylglycerol; DEA, docosatetraenoylethanolamide; DGLEA dihomogamma-linolenylethanolamide; DHEA, docosahexaenoylethanolamide; FDR, False Discovery Rate; MAG, monoacylglycerol; LNAA, large neutral amino acids; OEA, oleoylethanolamide; POEA, palmitoleylethanolamide.

of these clustered sets of metabolic markers were not statistically connected with the plasma concentration of the ayahuasca alkaloids. Connections between the metabolomics set and subjective ayahuasca effects and ayahuasca alkaloids therefore differed. The number of ceremonies, the number of years in Santo Daime, and the number of days since the previous ceremony were not statistically associated with any metabolic marker in this study.

Overall, a positive correlation of the cluster “alkaloids” with several endocannabinoids, lipids such as ceramides, hexosylceramides, LPCs, and organic acids was noted. In most of the cases, these markers did not present any relationship with the ayahuasca-induced subjective effects, except for the downstream metabolite DGLEA, which constitutes a nexus between ayahuasca alkaloids the elicited subjective effects.

A strong positive relationship with a set of metabolic markers, composed of tryptophan/LNAA, 5HIAA/tryptophan, 5HIAA/serotonin, and several monoacylglycerol (MAG) species, was observed for the 9 subscales of the ASC, with a special focus on the cluster termed “Effects A”. This cluster contained themes pertain to mystical/dissolution subscales: “the experience of unity”, “blissful state”, and “insightfulness”. Likewise, cluster “Effects B”, which comprised subscales of perceptual changes: “audio-visual synaesthesia”, “complex imagery” and “changed meaning of perception”; and cluster “Effects C”, which consisted of depersonalisation-based subscales: “disembodiment and “spiritual”, also presented a significant correlation, although weaker, with this set of markers. Conversely, no connections of this set of metabolic markers were observed with either the cluster of alkaloids or the dose. No associations were found for the number of days since the last consumption of ayahuasca, the number of ceremonies with ayahuasca or years in Santo Daime.

On the other hand, another set of metabolic markers composed of amino acids such as tyrosine, phenylalanine, isoleucine, and the sum of LNAAs was inversely correlated with the ASC clusters following a similar manner. The strongest associations were between tyrosine and the cluster “Effects A”, particularly for the subscale “the experience of unity”, where the higher the ratings, the lower the concentration of tyrosine.

The integrative network graph illustrates other minor connections observed for other metabolic groups with the ayahuasca-induced subjective effects and the ayahuasca alkaloids arm (Fig. 2). Interestingly, the ASC subscale “experience of unity” had the highest number of connections, followed by “elemental imagery” and “blissful state”. Most of the relationships come from the group of amino acid metabolism, and it is observed that these connections are not established with the plasma alkaloid concentrations. Remarkably, only DGLEA, among the endocannabinoids, is linked to both ayahuasca-induced effects and the alkaloids. Another large arm connects the ayahuasca-induced subjective effects with different species of ratios hexosylceramides/ceramides that, at the same time, link with the plasma concentrations of the ayahuasca alkaloids. Of the alkaloids, tetrahydroharmine had the highest number of connections, while the entire alkaloids cluster connected with all the endocannabinoids, certain LPCs, and energy metabolism metabolites.

3.3. Metabolic pathway enrichment

The metabolic pathway enrichment analysis mapped 11 out of the 31 significant metabolites with 243 items. Metabolites that were successfully found in the *Homo sapiens knowledge object* were valine, leucine, isoleucine, tyrosine, tryptophan, phenylalanine, glutamate, serotonin,

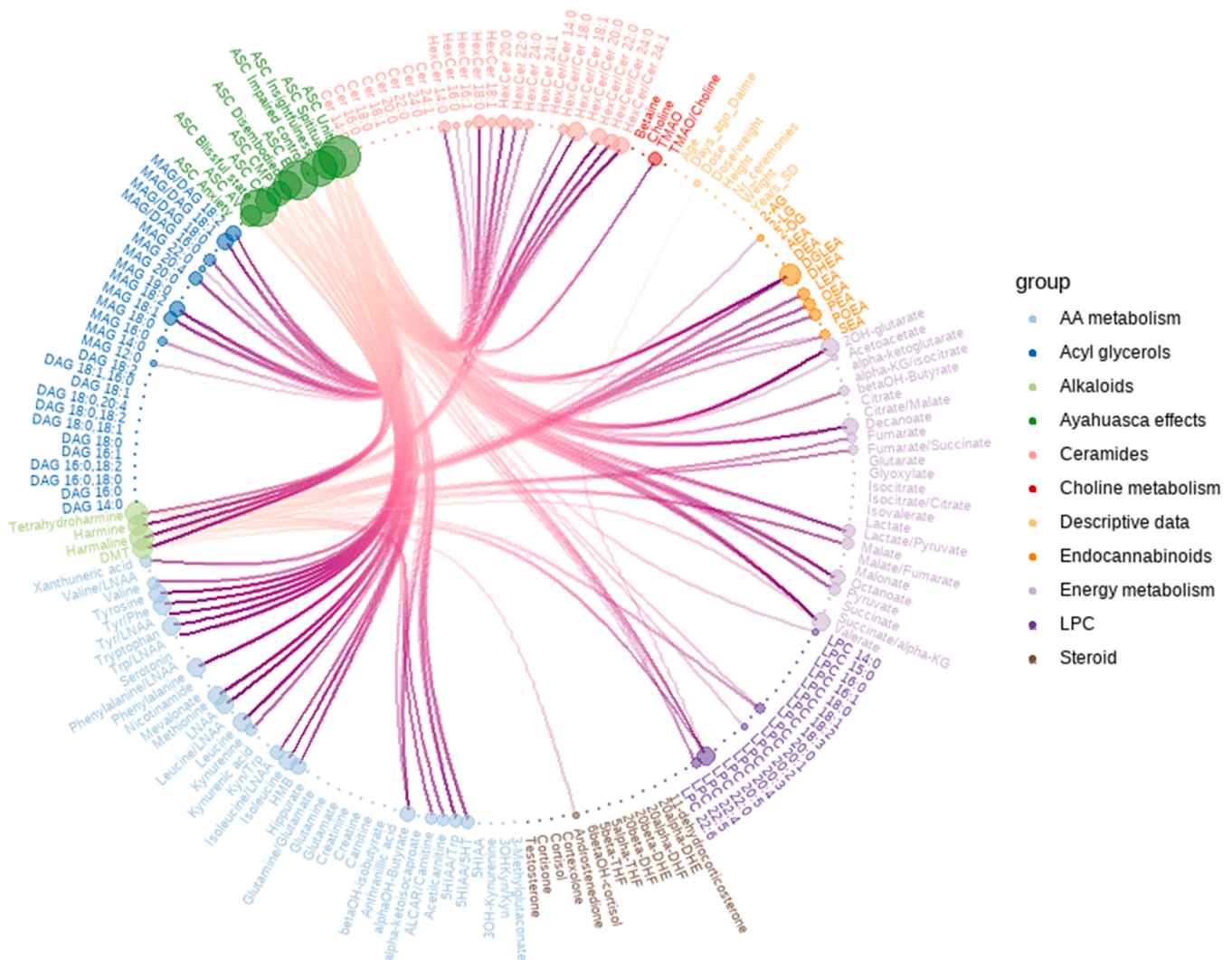


Fig. 2. Integrative network graph depicting correlations derived from regularized canonical correlation analysis between ayahuasca alkaloids, ayahuasca-induced subjective effects, metabolic markers and behavioural data based on a similarity score > 0.3. Nodes (circles) represent variables and are sized according to the number of connections. Abbreviations: 2-AG, 2-arachidonoyl glycerol; 2-LG, 2-linoleoyl glycerol; 2-OG, 2-oleoylglycerol; 5HIAA, 5-hydroxyindoleacetic acid; AEA, anandamide; AV, audio-visual synaesthesia; Cer, ceramide; CI, Complex Imagery; CMP, Changed meaning of perception; DAG, diacylglycerol; Days_ago_Daime, days since the last consumption of ayahuasca; Nr_ceremonies, number of ceremonies with ayahuasca; Years_SD, years in Santo Daime; DEA, docosate-traenoylethanolamide; DGLEA dihomoy-linolenylethanolamide; DHEA, docosahexaenylethanolamide; DMT, N,N-dimethyltryptamine; EI, Elemental imagery; FDR, False Discovery Rate; hexosylceramides; HexCer; MAG, monoacylglycerol; LNAA, large neutral amino acids; LPC, lysophosphatidylcholine; OEA, oleoylethanolamide; POEA, palmitoylethanolamide.

5HIAA, α -hydroxybutyrate and 2-oxobutanoate. Nevertheless, the algorithm was not able to detect any endocannabinoids in the *knowledge object* due to no entries for *Homo sapiens* on the date it was performed. The graphical representation of compounds, enzymes, reactions, modules, and pathways is shown in Fig. 3. The figure displays information on how the tested metabolites meet the suggested pathways. The graphical representation exhibits how the branched-chain amino acids (BCAAs, valine, leucine and isoleucine) and aromatic amino acids (AAAs, tyrosine, tryptophan and phenylalanine) pathways crosstalk for intracellular metabolites, reaching glutamate and serotonin neurotransmitters. The analysis framed these compounds in the modules of melatonin biosynthesis from serotonin and tryptophan, and dopamine biosynthesis from tyrosine through the dopaminergic synapse catalysed by the enzyme tyrosine 3-monooxygenase, among other enzymes related to AAAs. Other interconnected pathways are suggested by the propagation algorithm, based on previous entries in the KEGG, including synaptic vesicle cycle, GAP junction, mTOR signalling, amphetamine addiction, cocaine addiction and alcoholism.

4. Discussion

In the present study, we have identified the metabolic signature of the altered state of consciousness during an ayahuasca experience. This signature consisted of (i) alteration of the serotonin metabolism, (ii) alterations of endocannabinoids levels and (iii) dysregulation of amino acids balance.

Alterations in serotonin metabolism (identified by decreased levels of 5HIAA and the ratios 5HIAA/tryptophan and 5HIAA/serotonin) after ayahuasca consumption are in agreement with the MAO inhibition produced by β -carbolines [43]. This blockage of MAO would imply the accumulation of serotonin at the nerve terminals leading to serotonin syndrome [4,18]. Our results showed that this inhibition is positively correlated with most of the subjective effects of ayahuasca.

Ayahuasca consumption increased plasmatic levels of N-acyl ethanolamines such as AEA, whereas it decreased the levels of acylglycerols such as 2-AG. These results suggest a strong influence of ayahuasca on the peripheral endocannabinoid system, as has been suggested

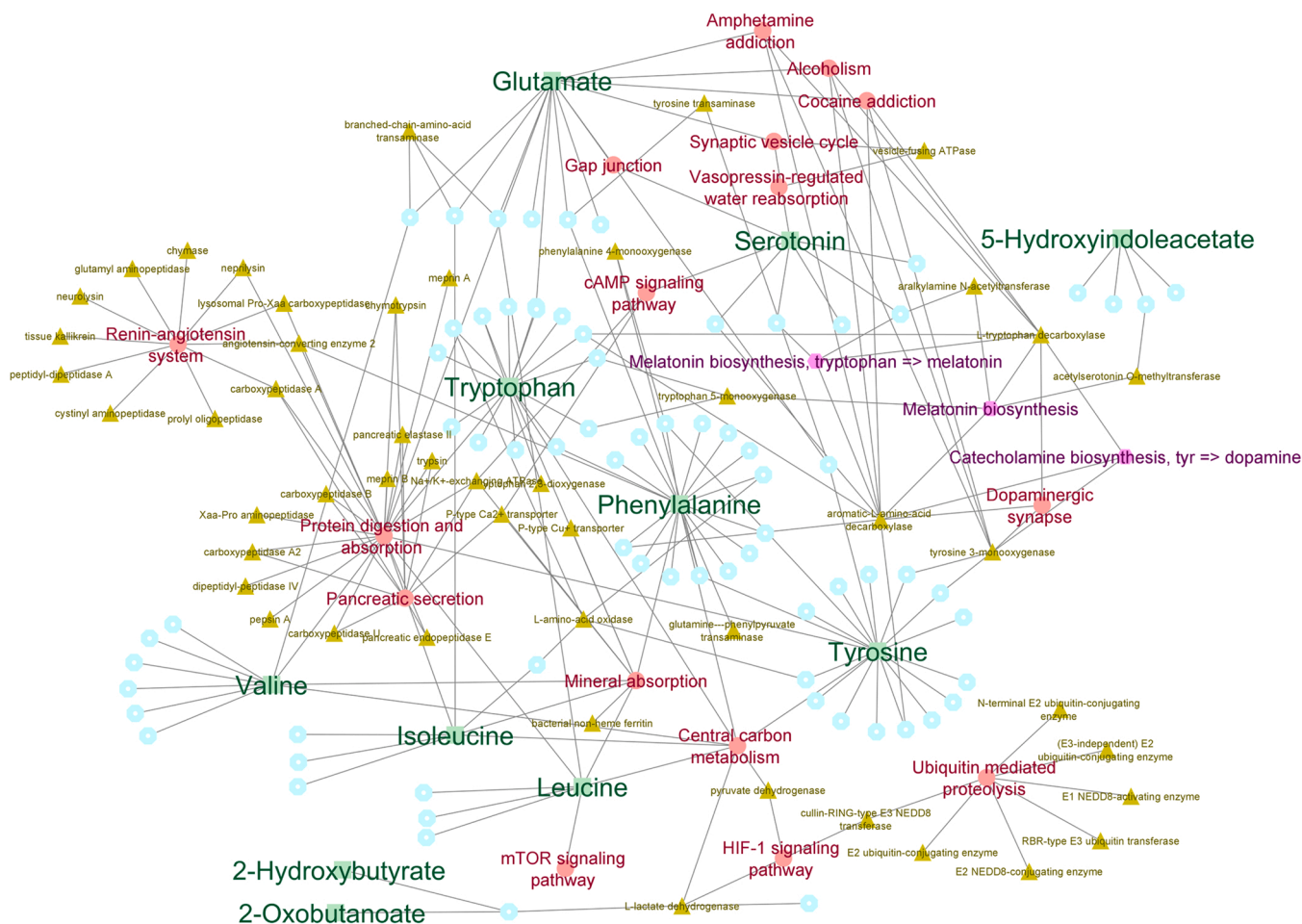


Fig. 3. Metabolic pathway enrichment analysis. The pathways (red), modules (pink), enzymes (yellow), and reactions (blue) are from KEGG database while the metabolites (green) that were significantly different after the ayahuasca consumption were used as input of the analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

previously (Rafael Guimarães [15]). From a mechanistic point of view, the modulation of these peripheral endocannabinoid levels can be explained by different factors. On one hand, chemical compounds from the β -carboline alkaloids family present in ayahuasca have been shown to inhibit the fatty acid amide hydrolase (FAAH) [48]. This enzyme is responsible for the degradation of N-acyl ethanolamines and leads to increased levels of these metabolites. On the other hand, DMT, the other component of ayahuasca preparations, is considered an agonist of 5-HT_{2A} receptors [59]. It has been demonstrated that the activation of these receptors can modulate 2-AG levels [49] although its impact on peripheral 2-AG levels is unknown. These are possibly direct mechanisms explaining the endocannabinoid modulation by ayahuasca, although it is very plausible that more complex mechanisms were involved in the modulation. For example, circulating concentrations of endocannabinoids have been associated with the circadian cycle and can be altered by stress, inflammation, pain, or sleep disturbances [29]. As our results showed that ayahuasca significantly increased the levels of some corticosteroids, this could be an alternative mechanism linking ayahuasca with endocannabinoids.

Our study focused on the analysis of peripheral metabolites. However, an important unresolved question is whether the levels of circulating metabolites such as the observed endocannabinoids are a biomarker for brain endocannabinoid signalling and how these are related to behaviour. Some previous studies are accumulating evidence that challenges this idea. For example, AEA concentration in cerebrospinal fluid is increased in particular situations without consistent changes in their circulating concentrations [30]. In addition, preclinical

studies showed no relationship between brain and plasma concentrations of 2-AG or AEA in rodent models of stress [28]. These discrepancies between peripheral and central levels of endocannabinoids might explain the lack of correlation between endocannabinoid levels and the subjective effects of ayahuasca. However, the endocannabinoid modulation could also be a possible mechanism underlying some of the therapeutic effects of ayahuasca. Indeed, both ayahuasca and endocannabinoid signalling have been proposed for the treatment of anxiety, depression or addiction (Rafael G. dos [61,70]). For example, increased anandamide levels have been linked to antidepressant effects [8] or anxiolysis [32]. Thus, ayahuasca could induce similar therapeutic effects through the enhancement of AEA levels that we have observed. In addition, agonism at 5-HT_{2A} receptors, which are a direct target of DMT, may modulate glutamatergic neurotransmission [41], leading to anxiolytic and antidepressant effects and enhancements of neuroplasticity [2,9,26,77]. Furthermore, 5-HT_{2A} receptors can form heteromers with the cannabinoid receptors in brain areas that regulate mood and emotion [76]. Nevertheless, more research is needed to clarify the link between the therapeutic effects induced by ayahuasca and the potential role of the endocannabinoid system.

Ayahuasca also modified the plasmatic levels of LNAAs. This result is in agreement with previous studies dealing with the administration of different drugs in animal models [46,72,73,85]. Since ayahuasca may ameliorate addiction (Rafael G. dos [61]), LNAAs might be key in this role, and thus, this might explain the potential association of LNAAs and pathways of addiction (alcoholism, and amphetamine and cocaine addiction) suggested in the enrichment analysis (Fig. 3). Ayahuasca

increased BCAAs, which may stimulate mTOR, a protein kinase involved in protein synthesis, synaptic plasticity, and neurotrophic signalling [37]. This was also proposed by the enrichment analysis (Fig. 3). Calvin and colleagues showed that psychedelics stimulate the mTOR signalling pathway, which leads to increased neuroplasticity [36]. Our results also showed that ayahuasca blunted the levels of tyrosine without altering the levels of tryptophan.

Remarkably, relative levels of tryptophan (evaluated by the ratio tryptophan/LNAA) positively correlated with subjective effects whereas levels of tyrosine (evaluated by tyrosine, tyrosine/phenylalanine and tyrosine/LNAA) and BCAAs showed a negative correlation with these effects. Both BCAAs and AAAs are precursors of monoamine neurotransmitters and thus, they play an essential role in central nervous system neurotransmitter biosynthesis [54]. Since BCAAs and AAAs share the same transporter to reach the brain, brain concentrations of neurotransmitters are influenced by the relative concentrations of these amino acids [16]. These results point out that higher levels of tryptophan and lower levels of tyrosine will cross the blood-brain-barrier suggesting an increase in serotonin levels and a decrease in the dopamine levels. Tryptophan has been associated with the stimulation of serotonin and serotonergic activity (X. [83]). Alterations in the tryptophan pathway provide insight into the connection between circulating tryptophan, serotonin and the 5-HT_{2A} activation by DMT [74] in the development of subjective effects (Fig. 2). A lower concentration of tyrosine points to an altered concentration of dopamine in the brain [6], as suggested by the enrichment analysis (Fig. 3). The inverse association of several ASC subscales of the subjective effects with tyrosine and all BCAAs and the positive association of the ratio tryptophan/LNAA, suggests a relevant role for serotonin in subjective ratings of “unity”, “blissful state” and “insightfulness”. Interestingly, it has been suggested that cluster A effects are related to enduring therapeutic outcomes [81]. We hypothesize that these subjective effects after the consumption of ayahuasca partially respond to the balance between circulating tryptophan and tyrosine in competition for transporters at the blood-brain barrier [16] and their conversion to neurotransmitters such as serotonin and catecholamines, respectively.

Remarkably, despite the strong association between LNAA concentrations and ayahuasca subjective effects, we found only a moderate association of peripheral LNAA with DMT concentrations (Fig. 1) and a mild association with harmine concentrations in blood. This result seems to indicate that the relationship between the trade-off in LNAA metabolism and the subjective effects may occur independently of the dose and the plasma concentration of the ayahuasca alkaloids.

A limitation of this study is that we did not control for the set and setting characteristics of the ceremonies. During the ayahuasca ceremonies, there are common preparational elements [57], which can affect the psychoactive response [27]. At that point, we cannot discard the contribution of these factors to the results of the present study. Moreover, the plasma alkaloid data do not represent the complete pharmacokinetic analysis but the concentration of a single timepoint. Future studies are needed in this area to better understand the relevance of the metabolic changes described in this study and the contribution of both the pharmacokinetics and the additional factors associated with the ceremony in the context of the therapeutic use of ayahuasca.

5. Conclusions

To our knowledge, this report is the first to show the impact on the human metabolome produced by a psychedelic drug, ayahuasca, a psychedelic drug whose effects are newly in the spotlight for the treatment of certain psychiatric illnesses. Using targeted metabolomics, plasma biomarkers (serotonin metabolism, N-acyl-ethanolamine and 2-acyl-glycerols endocannabinoids and LNAAs) were identified that can profile the neurophysiological effects. Likewise, integrated network analysis allowed us to explore ayahuasca-subjective effects and the connected metabolic compounds that might underlie the different

subjective effects, including the commonly described experience of “unity” that consumers report. In this sense, the biological mechanism involved an imbalance of circulating concentrations of the AAA such as tryptophan, phenylalanine and tyrosine. These compounds are precursors of serotonin and dopamine, norepinephrine and epinephrine. Importantly, such findings were not related to peripheral plasma drugs concentration. These findings suggest a crosstalk between the circulating LNAAs, their relationship with neurotransmitters, and the subjective experiences that could contribute to the described therapeutic effects of ayahuasca.

CRedit authorship contribution statement

Francisco Madrid-Gambin: Formal analysis, Writing – original draft preparation. **Alex Gomez-Gomez:** Methodology. **Arnau Busquets-Garcia:** Reviewing and Editing. **Noemí Haro:** Methodology. **Santiago Marco:** Supervision. **Natasha L. Mason:** Data curation and Resources. **Johannes T. Reckweg:** Methodology. **Pablo Mallaroni:** Methodology. **Lilian Kloft:** Methodology. **Kim van Oorsouw:** Investigation and Methodology. **Stefan W. Toennes:** Methodology. **Rafael de la Torre:** Resources. **Johannes G. Ramaekers:** Writing – reviewing and validation. **Oscar J. Pozo:** Conceptualization and Supervision.

Conflict of interest statement

The authors declare no potential conflicts of interest.

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