Supporting Information

Protective Effects of Chlorogenic Acid on Trimethyltin Chloride-Induced Neurobehavioral Dysfunctions in Mice Relying on Gut Microbiota

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EXPERIMENTAL SECTION

LC-MS analysis

The LC-MS analysis of phenolic metabolites, related organic acids, and SCFAs was carried out as previously described.[1-5] The analysis was performed on a UPLC-Orbitrap-MS-system consisting of a Thermo Q Exactive coupled to a Thermo Vanquish HPLC system equipped with an autoinjector and a binary solvent delivery system (Thermo, USA). For phenolic metabolites analysis, 50 mg brain tissue was homogenized with 200 μL extraction solution (Methanol: chloroform = 7:3) on ice for 30 min, then mixed well with 200 μL H_2O, following centrifuge at 12,000 rpm for 10 min at 4 ℃, remove the supernatant, and repeat the extraction once. Combined extractions were lyophilized, then added with 200ul of methanol, centrifuged at 12000 rpm for 10 min at 4 ℃, and collect the supernatant. For related organic acids and SCFAs analysis, the extraction method was the same as above, except for adding 10 μL EDC (0.1M) and 10 μL 3NPH (0.1M) per 40 μL extract solution for derivatization. Two complimentary chromatographic columns were used, including Water Acquity UPLC HSS T3 column (50 mm × 2.1 mm, 1.8 μm; Waters, USA) and Water Acquity BEH C18 column (50 mm × 2.1 mm, 1.8 μm; Waters, USA). For HSS T3, the mobile phase composed of phase A (0.1 % formic acid aqueous solution) and phase B (0.1 % formic acid acetonitrile) was at a flow rate of 0.3 mL/min. The gradient program was as follows, 0 min, 10% B; 2 min, 10% B; 6 min, 60% B; 8 min, 60% B; 8.1 min, 60% B; 12 min, 10%B. As of BEH C18, the mobile phase composed of phase A (0.1 % formic acid aqueous solution) and phase B (0.1 % formic acid acetonitrile) at a flow rate of 0.35 mL/min. The gradient program was as follows, 0 min, 10% B; 2 min, 10% B; 12 min, 90% B; 14 min, 90% B; 14.1 min, 10% B; 16 min, 10%B. All the column temperatures were 40 ℃. HRMS data were recorded on a Q Exactive hybrid Q–Orbitrap mass spectrometer equipped with a heated ESI source (Thermo Fisher Scientific) utilizing the SIM MS or Fullms-ms2 acquisition methods. The ESI source parameters were set as follows: spray voltage, -2.8 kV/3.0 kV; sheath gas pressure, 40 arb; aux gas pressure, 10 arb; sweep gas pressure, 0 arb; capillary temperature, 320℃; and aux gas heater temperature, 350℃. We have performed validation experiments

such as Retention time, Linear equations, Correlation coefficient, Linear Range, LOQ, see the Table S1 for details. Standard products used for the quantitative study were purchased from Sigma Co. (St. Louis, USA).

Table S1. Details for quantification of phenolic metabolites and related organic acids.

Compounds	Retention time (min)	Calibration	Fit	Range ng/mL	LOQ ng/mL
Chlorogenic acid	4.900	Y = 9.427e4X	0.9983	10-5000	10
Neochlorogenic acid	3.820	Y = 1.357e5X	0.9989	10-5000	10
Cryptochlorogenic acid	5.080	Y = 1.409e5X	0.9972	10-5000	10
3,4-Dicaffeoylquinic acid	6.650	Y = 7.198e4X	0.9919	10-5000	10
4,5-Dicaffeoylquinic acid	6.900	Y = 8.048e4X	0.9973	10-5000	10
3,5-dicaffeoylquinic acid	6.770	Y = 7.123e4X	0.9978	10-5000	1
Phe(L-Phenylalanine)	1.940	Y = 5.93e4X	0.9942	1-5000	1
Protocatechualdehyde	4.900	Y = 2.81e5X	0.9971	5-3000	5
Catechin	5.100	Y = 1.133e5X	0.9963	0.5-300	0.5
Vanillic acid	5.500	Y = 1.168e5X	0.9975	5-3000	5
Caffeic acid	5.530	Y = 1.484e5X	0.9980	5-3000	5
Syringic acid	5.710	Y = 1.084e5X	0.9959	0.5-300	0.5
L-Epicatechin	5.740	Y = 1.435e5X	0.9990	0.5-300	0.5
p-Hydroxycinnamic Acid	6.380	Y = 5.249e4X	0.9970	5-3000	5
4-Hydroxybenzoic acid	6.420	Y = 2.197e4X	0.9950	5-3000	5
Vanillin	6.420	Y = 1.157e5X	0.9954	5-3000	5
Syringaldehyde	6.620	Y = 1.832e5X	0.9961	5-3000	5
4-Hydroxy-3,5-dimethoxycinnamic acid	6.680	Y = 1.333e5X	0.9941	5-3000	5

Ferulic Acid	6.680	Y = 9.3e4X	0.9962	5-3000	5
Hydrocinnamic acid	8.120	Y = 9.846e4X	0.9958	5-3000	5
Trans-Cinnamic acid	8.160	Y = 8.448e4X	0.9966	5-3000	5
3,4-Dihydroxybenzoic acid	3.470	Y = 8.38e4X	0.9987	0.5-5000	5
Gallic acid	1.710	Y = 2.886e3X	0.9982	1-5000	1
3-Hydroxy-4-methoxycinnamic acid	5.190	Y = 2.009e5X	0.9975	0.5-1000	0.5
Dihydrocaffeic acid	5.250	Y = 1.812e5X	0.9966	1-1000	1
3-Hydroxy-3-phenylpropionic acid	6.190	Y = 1.553e5X	0.9982	0.5-1000	0.5
Hippuric acid	6.830	Y = 9.619e4X	0.9975	0.5-1000	0.5
Lactic acid	3.560	Y = 1.11e5X	0.9988	50-20000	50
D(-)-Tartaric acid	7.390	Y = 3.067e5X	0.9966	50-15000	50
Itaconic acid	8.890	Y = 4.431e4X	0.9972	50-20000	50
Succinic acid	8.510	Y = 1.489e4X	0.9918	50-15000	50
Malonic acid	8.510	Y = 1.281e5X	0.9956	50-15000	50
Glutaric acid	8.910	Y = 7.771e3X	0.9949	50-15000	50
Adipic acid	9.170	Y = 7.034e3X	0.9963	50-20000	50
Benzoic acid	9.340	Y = 1.912e5X	0.9941	50-5000	50
Oxalic acid	9.600	Y = 1.6e4X	0.9992	50-20000	50
Pimelic acid	9.650	Y = 4.911e3X	0.9966	50-20000	50
DL-isocitrate	10.100	Y = 1.101e3X	0.9978	100-20000	100
Salicylic acid	10.140	Y = 6.213e4X	0.9966	50-20000	50
Citric acid	10.570	Y = 1.647e3X	0.9980	100-20000	100
Octanoic acid	12.750	Y = 5.622e4X	0.9934	50-20000	50
Shikimic acid	2.170	Y = 4.358e4X	0.9976	50-5000	50
Quinic acid	2.330	Y = 8.723e4X	0.9935	50-10000	50
Malic acid	8.030	Y = 1.077e5X	0.9958	50-2000	50

Fumaric acid	9.310	Y = 1.907e5X	0.9957	50-10000	50
Maleic acid	9.450	Y = 2.764e4X	0.9988	50-20000	50
Isobutyric acid	7.370	Y = 4.577e4X	0.9964	50-10000	50
Acetic acid	2.830	Y = 1.072e5X	0.9983	50-20000	50
4-Methylvaleric acid	10.430	Y = 6.112e4X	0.9943	50-20000	50
Isovaleric acid	8.930	Y = 5.975e4X	0.9954	50-20000	50
Valeric acid	9.200	Y = 6.195e4X	0.9964	50-20000	50
Propionic acid	5.890	Y = 4.038e4X	0.9950	50-15000	50
Butyric Acid	7.560	Y = 6.637e4X	0.9986	50-20000	50
Hexanoic acid	10.600	Y = 6.659e4X	0.9966	50-20000	50

Neurotransmitter analysis was carried out as previously described.[6-9] This analysis was performed on a UPLC-MS-system consisting of an AB SCIEX coupled to a Waters ACQUITY UPLC system equipped with an autoinjector and a binary solvent delivery system (Thermo, USA). The sample was accurately weighed in a 2 mL EP tube, 600 μL of 10 % formic acid methanol-H_2O (1:1.V/V) solution was added, then vortexed for 30 s. The sample was placed in a tissue grinder and ground at 60 Hz for 90 s. The supernatant was centrifuged at 12000 rpm for 5 min at 4 ℃, 100 μL of the supernatant was collected, and 100 ppb of isotope was added accurately. Water Acquity BEH C18 column (50 mm × 2.1 mm, 1.8 μm; Waters, USA) was used at a column temperature at 40℃. The mobile phase composed of phase A (10% methanol solution contains 0.1 % formic acid) and phase B (50% methanol solution contains 0.1 % formic acid) was at a flow rate of 0.4 mL/min. The gradient program was as follows, 0-1 min, 20%-100% B; 1-7 min, 100% B; 7-7.5 min, 100-20% B; 7.5-11 min, 20% B. Mass spectra were obtained using an Acquity UPLC electrospray ionization (ESI). The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. The ESI source parameters were set as follows: capillary voltage was set to 5500 V, collision gas to 6 psi, air curtain gas to 30 psi for, and both atomization gas and auxiliary gas to 50 psi, ion source temperature to 150 ◦C. Nitrogen, obtained from a nitrogen generator (99.93 %) was

used for desolvation. MRM transitions, cone voltage and collision energy showed in Supplementary materials S3. The collected data is analyzed by TargetLnyx soft. We have performed validation experiments such as Retention time, Linear equations, Correlation coefficient, Linear Range, LOQ, see the Table S1 for details. Standard products used for the quantitative study were purchased from Sigma Co. (St. Louis, USA).

Table S2. Details for quantification of neurotransmitters.

Compounds	Retention time (min)	Calibration	Fit	Range ng/mL	LOQ ng/mL
GABA	4.278	y=0.01062x + 0.007717	0.9955	1-250	1
HisA	3.788	y=0.3402x - 0.01394	0.9965	0.5-25	0.5
PA	5.233	y=0.008861x + 0.003503	0.9902	2.5-625	2.5
TyrA	5.8	y=0.05848x + 0.01049	0.9902	0.5-125	0.5
Ach	4.391	y=0.438x + 0.0009176	0.9979	0.2-10	0.2
Gln	4.352	y=0.02059x + 0.05742	0.9951	1~250	1
Glu	4.429	y=0.01895x + 0.07221	0.9907	2~400	2
DA	4.905	y=0.0234x + 0.01653	0.9935	1~125	1
His	4.043	y=0.07489x + 0.05975	0.9902	2~200	2
TrpA	10.83	y=0.1793x + 0.005723	0.9939	0.2~20	0.2

NE	4.3	y=0.01728x + 0.001421	0.9929	2~200	2
5-HT	6.472	y=0.07062x + 0.006734	0.9931	1~100	1
Tyr	6.206	y=0.01564x + 0.02409	0.993	2.5~250	2.5
E	4.41	y=0.06843x + 0.002544	0.9907	0.5~50	0.5
KynA	11.47	y=0.00426x + 0.003748	0.9929	2~250	2
5-HIAA	11.06	y=0.02916x + 0.006752	0.9916	1~200	1
DOPA	5.385	y=0.02371x + 0.01353	0.9919	2.5~125	2.5
Trp	10.92	y=0.04886x + 0.02733	0.9919	0.5~100	0.5
XA	11.24	y=0.05529x - 0.001483	0.9948	2~200	2
Kyn	8.506	y=0.01038x + 0.004458	0.992	2~400	2
VMA	8.263	y=0.004944x + 0.02613	0.9929	25~1000	100
5-HTP	7.297	y=0.02958x + 0.001283	0.9937	0.5~50	1
MT	13.52	y=0.3564x + 0.004971	0.9929	0.1~20	0.1

Bioinformatic analysis

Protein target prediction was performed using SEA database (https://sea.bkslab.org/), protein association network analysis was performed using STRING (https://string-db.org/) and Cytoscape software 3.8.2 (A software environment for integrated models of biomolecular interaction networks.). Cluster analysis was performed using Clustvis (ClustVis: a web tool for visualizing clustering of multivariate data (BETA) (ut.ee)).

Western Blot analysis

Hippocampus tissue homogenates were obtained using RIPA buffer (P0013B, Beyotime) with a 1% protease inhibitor cocktail (4693116001, Roche). The homogenates were centrifuged at 20 000 g for 30 minutes at 4°C, and the supernatants were collected as protein samples. A protein assay kit (P0010, Beyotime) was used to determine the protein concentrations. The samples (50 μg/sample) were solubilized with 5× SLB (P0015, Beyotime), heated at 95°C for 15 minutes, and then subject to 12% SDS-PAGE. Following electrophoresis, the samples were transferred onto polyvinylidene fluoride membranes (1620177, Bio-Rad). The membranes were blocked in 5% nonfat powdered milk in PBS for 2 hours at room temperature. After appropriate antibodies were used for recognition, the immunoreactive bands were quantified using ImageJ software. The information of primary antibodies and secondary antibodies is shown in Table S3.

Immunofluorescence analysis

Mouse brain sections were pretreated in 0.3% H2O2 in PBS for 10 minutes and then incubated in 1% Triton X-100/PBS solution for 30 minutes. All sections were blocked with 10% normal donkey serum and 0.3% Triton X-100/PBS for 2 hours at room temperature, followed by incubation with appropriate antibodies. The staining was visualized with Alexa Fluor conjugated secondary antibodies. Nuclei were stained with DAPI (C1005, Beyotime). The sections were finally mounted on glass slides. All

sections were examined by a confocal laser scanning microscope (LSM 780, Zeiss). The information of primary antibodies and secondary antibodies is shown in Table S3.

Table S3. Antibodies

Resource	Source	Identifier
PSD 95	Abcam	ab238135
Synaptophysin	Abcam	ab32127
TLR4	Abclone	A5258
p-NFκB p65 (ser536)	Cell Signaling Technology	13346
NFκB p65	Cell Signaling Technology	8242
p-c-Jun (ser73)	Cell Signaling Technology	3270
c-Jun	Cell Signaling Technology	9165
p-JNK(Thr183/Tyr185)	Cell Signaling Technology	9255
JNK	Cell Signaling Technology	9252
Caspase 9	Cell Signaling Technology	9508
Caspase 3	Cell Signaling Technology	9664
APP	Invitrogen	MA1-25489
GFAP	Abcam	ab7260
IBA-1	Abcam	ab178847
TNF-α	Abcam	ab215188
IL-1β	Cell Signaling Technology	CS12242
ACTB	Sigma	A5441
GAPDH	Abcam	ab8245
β-Tublin	Cell Signaling Technology	2128
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP	Beyotime	A0208
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP	Beyotime	A0216
Alexa Fluor 568-conjugated secondary antibodies	Invitrogen	A11011
Alexa Fluor 488-conjugated secondary antibodies	Invitrogen	A32814

RESLUTS

Table S4. Concentrations (ng/g) of phenolic metabolites and related organic acids in mouse hippocampus of different groups.

Compounds	**Treatments**		
	Control	**TMT**	**TMT+CGA**
Neochlorogenic acid	2.09±0.66a	1.73±0.62a	1.51±0.39a
Chlorogenic acid	57.30±23.73a	44.02±17.71a	57.11±51.58a
Cryptochlorogenic acid	77.11±44.67a	66.45±65.68a	57.33±15.68a
3,4-Dicaffeoylquinic acid	646.37±618.61a	560.77±636.73a	175.45±50.83a
3,5-dicaffeoylquinic acid	786.77±737.27a	669.66±703.69a	222.75±50.23a
4,5-Dicaffeoylquinic acid	487.74±354.84a	304.98±297.58a	426.41±137.34a
Gallic acid	5.74±2.21a	5.60±3.58a	3.17±0.72a
Phe(L-Phenylalanine)	47.10±8.80c	62.20±5.52a	58.00±3.24ab
3,4-Dihydroxybenzoic acid	1.59±0.70a	1.45±0.67a	0.83±0.20a
Protocatechualdehyde	40.56±4.96a	38.72±4.20a	41.73±4.87a
Catechin	0.09±0.07a	0.06±0.05ab	0.01±0.01bc
Vanillic acid	3.27±1.03c	6.47±3.87a	5.71±0.93ab
Caffeic acid	5.11±3.60a	4.74±3.34a	2.73±1.30a
Syringic acid	2.94±1.91b	4.43±2.39ab	6.84±0.76a
L-Epicatechin	0.64±0.57a	0.50±0.53a	0.12±0.02a
p-Hydroxycinnamic Acid	8.10±3.25a	7.96±1.57a	9.99±4.15a
4-Hydroxybenzoic acid	66.39±6.24c	77.25±6.54ab	78.74±7.87a
Vanillin	73.57±6.79b	82.26±6.62ab	83.63±7.70a
Syringaldehyde	5.80±5.65b	14.73±14.40b	46.33±5.91a
4-Hydroxy-3,5-dimethoxycinnamic acid	2.14±1.40a	2.05±1.47a	1.55±0.77a
Ferulic Acid	23.82±23.90a	10.70±3.25a	20.54±17.72a
Hydrocinnamic acid	7.57±1.63a	7.27±1.50a	8.68±4.21a
Trans-Cinnamic acid	3.41±2.51a	3.23±2.28a	2.64±0.65a
3-Hydroxy-4-methoxycinnamic acid	10.91±2.69a	11.87±2.64a	12.62±3.11a
Dihydrocaffeic acid	0.00±0.00a	0.00±0.00a	0.00±0.00a
3-Hydroxy-3-phenylpropionic acid	0.51±0.15a	0.50±0.15a	0.52±0.10a
Hippuric acid	8.42±6.63a	3.00±0.33b	3.70±1.52b
Shikimic acid	1.19±1.74a	0.49±1.10a	0.00±0.00a
Quinic acid	24.23±15.83a	17.10±19.15a	19.82±18.48a
Lactic acid	1191187.23±71663.74a	1068803.62±119048.26a	1115283.60±195050.93a
Itaconic acid	178.05±9.52a	148.83±30.02a	164.84±44.34a
D(-)-Tartaric acid	33.42±4.83a	29.24±11.57a	40.70±19.54a
Malic acid	192481.85±9875.16a	172264.70±20111.87a	179985.95±35044.33a
Succinic acid	32627.35±3255.87a	30278.86±10425.68a	27649.17±5425.58a
Malonic acid	1003.24±114.26a	994.63±119.16a	961.14±213.09a

Glutaric acid	31078.48±4838.08a	25588.87±3665.18a	30521.62±7790.88a
Adipic acid	780.23±173.68a	729.43±203.92a	784.10±203.55a
Benzoic acid	55.67±9.37a	53.97±5.09a	67.63±16.33a
Oxalic acid	55037.27±6072.90a	44475.69±5446.11b	59849.60±7177.52a
Pimelic acid	715.42±154.08a	695.31±24.72a	734.81±302.03a
DL-isocitrate	10495.82±2083.90b	10595.87±1114.63ab	15741.11±6223.93a
Salicylic acid	11.75±7.56a	20.10±7.82a	17.59±14.20a
Citric acid	237967.20±45293.22b	260186.36±15210.32ab	339349.30±103973.20a
Octanoic acid	253.67±78.46a	294.10±35.34a	317.00±103.02a
Fumaric acid	24359.91±1295.14a	27060.48±2733.68a	26915.21±5198.01a
Maleic acid	131299.36±5655.70a	119785.26±25775.97a	108739.21±19390.15a

Values are expressed as means ± standard deviation. Different superscripts (a-c) in the same row indicate significant difference ($p < 0.05$).

Table S5. Concentrations (ng/g for HisA, Ach, DA, NE, 5-HT, E, 5-HIAA, XA, Kyn, VMA, 5-HTP; μg/g for GABA, Gln, Glu, His, Tyr, Trp) of neurotransmitters in mouse hippocampus of different groups.

Compounds	**Treatments**		
	Control	**TMT**	**TMT+CGA**
HisA	2.35±0.31a	2.54±0.41a	2.11±0.23a
Ach	29.76±2.15b	43.76±7.10a	31.91±4.15b
DA	1.07±2.39a	0.00±0.00a	5.32±7.65a
NE	129.97±27.54a	149.44±36.29a	126.58±33.05a
5-HT	0.00±0.00a	0.16±0.30a	0.03±0.08a
E	6.12±0.88a	7.37±0.89a	6.13±1.25a
5-HIAA	63.08±15.46a	75.38±19.35a	62.03±22.62a
XA	9.58±14.25a	0.00±0.00a	0.00±0.00a
Kyn	0.00±0.00b	3.09±2.80a	0.09±0.20b
5-HTP	2.11±0.92a	1.60±0.67a	1.62±1.37a
GABA	998.53±67.25a	984.35±113.33a	1000.54±105.99a
Gln	748.66±71.38a	913.75±114.63a	926.89±146.72a
Glu	741.55±160.68a	699.50±131.22a	690.50±72.38a
His	42.63±4.56a	39.23±3.51a	40.81±9.51a
Tyr	55.11±1.75a	47.65±8.20a	53.47±9.91a
Trp	17.15±1.12a	16.91±2.77a	16.80±3.10a

Values are expressed as means ± standard deviation. Different superscripts (a–b) in the same row indicate significant difference ($p < 0.05$).

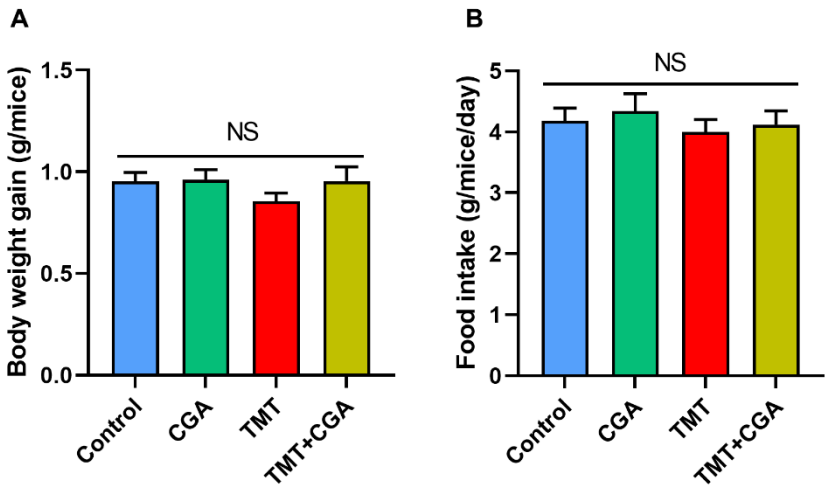

Figure S1 (A) Body weight gain and (B) food intake of mice with different treatments (n=16 per group) during the experiment. The values are represented as the mean ± SEM. NS, no significance.

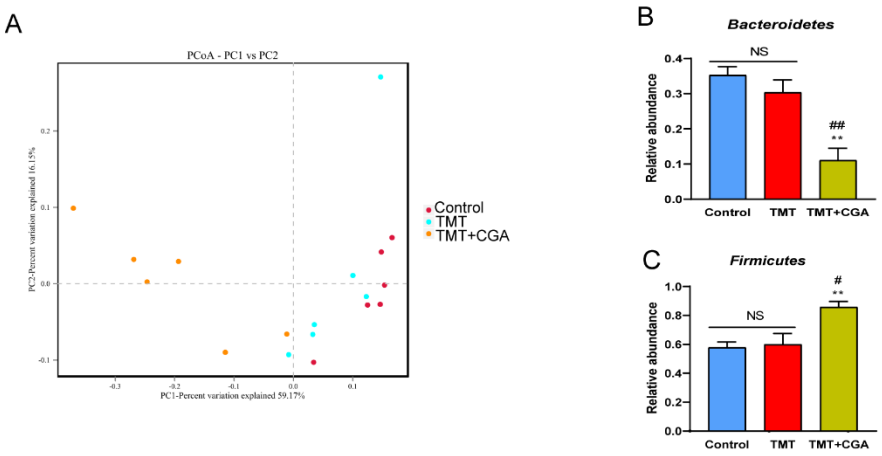

Figure S2.

(A) Principal coordinates (PCoA） analysis based on weighted_unifrac algorithm. (B) Relative abundance of *Bacteroidetes* in mice with different treatments. (C) Relative abundance of *Firmicutes* in mice with different treatments.

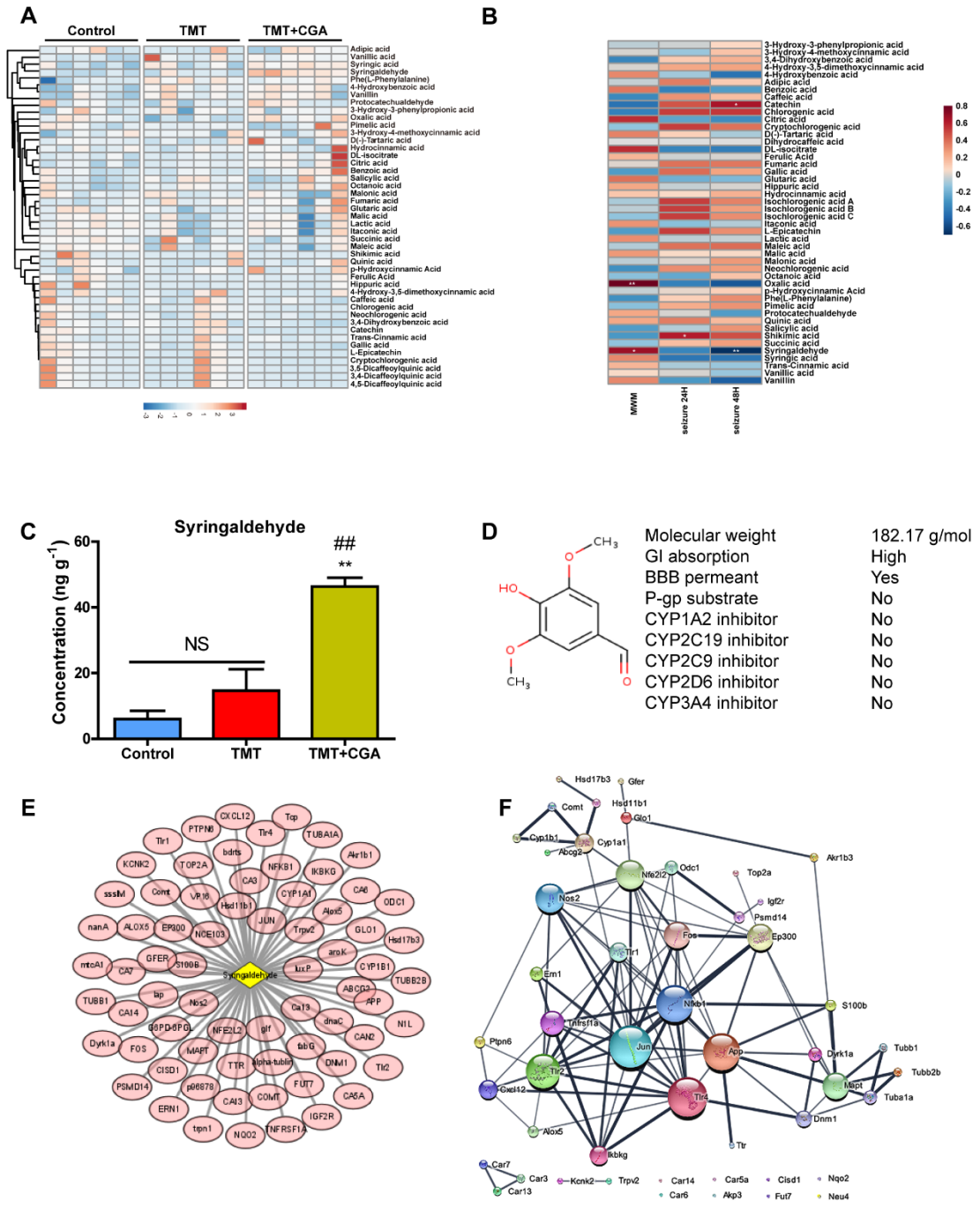

Figure S3. Target metabolomics and bioinformatics analysis of CGA metabolites in the mouse brain. (A) Heatmap of CGA metabolites in the hippocampus of mice with different treatments (n=6, per group). Unit variance scaling was used. (B) Correlation analysis between CGA metabolites and neuronal damages. (C) Concentration of syringaldehyde in the hippocampus of mice with different treatments (n=6 per group).

(D) Pharmacokinetics of syringaldehyde by SwissADME (http://www.swissadme.ch/index.php). (E) Protein targets prediction of syringaldehyde by SAE database (https://sea.bkslab.org/). (F) Protein association network analysis by STRING (https://string-db.org/) and Cytoscape software. Correlation analysis was performed using R version 3.6.1 (spearman method), *$p < 0.05$, **$p < 0.01$. The values are represented as the mean ± SEM. **$p < 0.01$ vs control group; ##$p < 0.01$ vs TMT group, NS, no significance.

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