Supporting Information

Bidirectional Microbiome-Gut-Brain-Axis Communication Influences Metabolic Switch-associated Responses in the Mosquito *Anopheles culicifacies*

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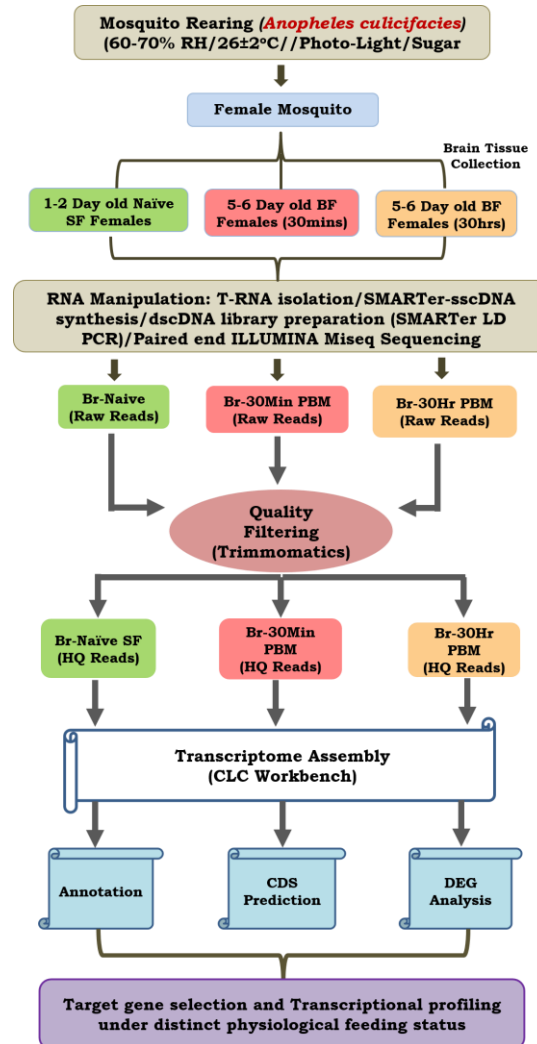†These authors contributed equally to this work.

Figure S1. A technical overview to decode the hard-wired genetic structure of brain system of *Anopheles culicifacies* mosquito.

Table S1. Validation DGE data.

Gene Name	Transcriptome data/DEG	Real Time Validation
serine/threonine-protein kinase (STPK)	Br_30hr = -1.2-Fold Down Br-30min = - 5.7-Fold Down	10hr = Upregulation 30min and 30hr = Downregulation
Neurotransmitter gated ion channel (NTGIC)	No Change	No Change
Glycine receptor (Gly Receptor)	No Change	No Change
Gamma-aminobutyric acid type B receptor (GABA Receptor)	Br_30hr = -1.1-fold down Br_30min = -0.27-Fold Down	10hr = Non-significant Upregulation 30min and 30hr = Downregulation
Glutamate receptor (Glut Receptor)	No Change	No Change
phosphatidylinositol-Phosphilipase C gamma (PLC)	Br-30hr = -0.2-Fold Down Br-30min = -0.13-Fold Down	10hr = Upregulation 30min and 30hr = Downregulation

Table S2. List of Primer Sequence used in the study.

Sl. No.	Primer Name and Sequence
1.	Actin_Fw: 5' TGCGTGACATCAAGGAGAAG 3' Actin_Rev: 5' GATTCCATACCCAGGAACGA 3'
2.	Ac_PyruvateKinase_Fw: 5' CGCACTTGATCTCCAAGTAT 3' Ac_PyruvateKinase_Rev: 5' TTCCAGCCAGTAACAACAA 3'
3.	Ac_Solute Carrier 7_Fw: 5' TCAATAGCTCCGAAATCAGT 3' Ac_Solute Carrier 7_Rev: 5' TGATAACGAACAGCAAGACA 3'
4.	Ac_AATransporter_Fw: 5' CAATGCCTATGGTTACAGGT 3' Ac_AATransporter_Rev: 5' GCTGGTAAGTGTCCTTCTTG 3'
5.	Ac_TrehalaseTransporter_Fw: 5' CGATGGGACTGTACTTCTTC 3' Ac_TrahalaseTransporter_Rev: 5' GTCTAGATCGGCGAAAAAC 3'
6.	Ac_PGC1_Fw: 5' ACCTTACGGTAAAATCGTCA 3' Ac_PGC1_Rev: 5' GTACGGTAGCTGATGTTCGT 3'
7.	Ac_OxoglutarateDHS_Fw: 5' GCAACTACTTCCATCTGCTC 3' Ac_OxoglutarateDHS_Rev: 5' GAGCCTTCAACAAGTCGTAA 3'
8.	cGMP PK_Fw: 5' GCGTTTGATTATCTGCACTC 3' cGMP PK_Rev: 5' AAGGACTCCAAGTGACCAGT 3'

9.	GlutamateR_Fw: 5'AGTGGTATCAACGCAGAGTG 3' GlutamateR_Rev: 5' GAGTTTAAGCACTGCTCCAC 3'
10.	Glycine R_Fw: 5' GATACTGCCACTACCTCGTC 3' Glycine R_Rev: 5' CTTGGAGACCGAATTGAATC 3'
11.	GABA R_Fw: 5' CAGAACGAAGAAGGCTACTC 3' GABA R_Fw: 5' AGTATCCACGCATACTCAGC 3'
12.	ARMAA_Decarboxylase_Fw: 5'GGTAACCAAGTCCTTCAGTG 3' ARMAA_Decarboxylase_Rev: 5' TAGAACAGACGACCTCGAAC 3'
13.	ILP1_Fw: TCCACTACATGGAAAACTCC ILP1_Rev: GTCATCAGTGCCTGGTAGAT
14.	ILP3_Fw: TAGCAATGATGAGTGGATGA ILP3_Rev: ACAACACTCCTCTACGATGC
15.	Leukokinin_Fw: AAACATCGCATAGCAGAGAT Leukokinin_rev: TCAGATAATCCTGCACCATA
16.	NRY_Fw: TACTGTACGGCTGGTTGAAT NRY_Rev: TTAGTTCCGGCAGTGTTC
17.	OEH_Fw: GACAAGAATGCGGTGATAAT OEH_Rev: CGTTGCTGTAGTAATCGAAG
18.	DH44R1_Fw: CTCGAAATAGAATGCTCCTG DH44R1_Rev: AGATGACGATGAGGTAGGTG

19.	LKR_Fw: AAGAGGGAACACGACAAAC LKR_Rev: GCTCGATATAATTGGTGGTC
20.	DH44_Fw: AACGAACAGGAAGATCTCAA DH44_Rev: ATACCGTAGACGTACCGTGA
21.	CCHAR2_Fw: CCACTCCGAAAACTACAGAC CCHAR2_Rev: GTGGCAGGAAGTAGTAAACG
22.	V-Type ATPase_Fw: TTACATGTACACCGATTTGG V-Type ATPase_Rev: GACTTCATCAGACGTGACAG
23.	DopR_Fw:5' GTTATGGGCGTGTTTATTGT 3' DopR_Rev:5' GCTGGTACTTGCGTCTTATC 3'
24.	AKT Kinase_Fw: GATGGAGGAGGTAAAGTTCC AKT Kinase_Rev: GAACTCACGGTCGAAGTAAC
25.	CYP314A1_Fw: GAGATTGCGCAAGAATTTAG CYP314A1_Rev: GGAAGTTGTCCTCACTCTGA
26.	PTTH_Fw: CTTCACCTCTGAATTGCTTC PTTH_Rev: ACAAGAAGACGGGTACTGTG
27.	KDNaCaExchanger_Fw: GTGAGATGGGTATCAGCAAC KDNaCaExchanger_Rev: CTTCCAATCAAGTTTGAAGC

28.	GABA ClCh_Fw: 5' GGAAGGTGTTTGGTAAGTCA 3' GABA ClCh_Rev: 5' GGTGATCGTGTTCGAGTAAT 3'
29.	PLC_Fw: 5' TGGATTCGTCCAACTATCAT 3' PLC_Rev: 5' TTCACGATCACCTCGTTC 3'
30.	PI-4Kinase_Fw: 5' ACATCATCTCCTCACTGTCC 3' PI-4Kinase_Rev: 5'GTGTGCCACTGTTGTAATCA 3'
31.	ST ProteinKinase_Fw: 5'TTTATAGTGCCGTGTGTTGA 3' ST ProteinKinase_Rev: 5'CTTAATGTGGAACCGATCAT 3'
32.	Trehalase_Fw: 5' GAAGAGGACAAACAGGACTA 3' Trehalase_Rev: 5' GTTCCGGTAACCATAGAAC 3'
33.	5-HT Receptor_Fw: 5' ATGATCTCGCGTAACTCCTC 3' 5-HT Receptor_Rev: 5' ATCGGATTGACCAGACTGC 3'
34.	TOR_Fw: GTAGAATGTTGGTGGTCGAT TOR_Rev: ACCATCTGCTAGGTTATTGC
35.	Octopamine Receptor_Fw: 5'CTACTGGCGGATCTATCGGG 3' Octopamine Receptor_Rev: 5' TGGTGGAAGGCTGTGTTTTG 3'
36.	Calcitonin R_Fw: AATAGAATGCTGGATGAACG Calcitonin R_Rev: GGACGAAACGGTGTAAGTAT

37.	NTGated IonCh_Fw: 5' ACGTTTCGAAAGTCAAACAC 3' NTGated IonCh_Rev: 5' GCTGTAGAATGCACAAATGA 3'

Table S3a. Annotation kinetics of RNA-Seq data.

Molecular Features	Ac-Br-Naive	Ac-Br-30M PBM	Ac-Br-30Hr PBM
Total No. of Raw Reads	5268211	3947521	3760078
Total No. of Contigs	32118	32984	38512
Total Transcripts	9460	9146	7387
Total BLASTx hits (NR)	8,668 (~91%)	8,336 (~91%)	6,548 (~88%)
Transcripts with GO Match			
Molecular Function	4773	4556	3575
Biological process	4446	4299	3381
Cellular component	2523	2424	1888

Table S3b. Percentage of differentially expressed transcripts.

Sample	No. of Transcripts	Transcripts showing Differential gene Expression (DGE)	Upregulated Transcripts	Downregulated Transcripts	Percentage of Transcripts showing DGE
Ac_Br_naive vs Ac_Br_30min	(9460 + 9146) = 18606	Total - 4747 Significant – 3183 Not significant - 1564	622 (3%)	2110 (11%)	14% CDS show differential expression
Ac_Br_Naive vs Ac_Br_30hr	(9460 + 7387) = 16847	Total -3966 Significant – 3174 Not significant - 792	482 (2%)	2469 (14%)	16 % show differential expression

Table S4. Comparative alpha diversity indices estimation of gut-bacterial population of naïve sugar fed and blood fed mosquito *An. culicifacies*.

Sample	Taxonomy Rank: Class	
	Shannon index (H)/(h/LN(N))	Simpson index
Ac_SF_MG	3.233/0.6802	0.9081
Ac_BF_MG	2.815/0.6424	0.888

Table S5. Quantitative estimation of 13 different neurotransmitters in the brain and the gut of mosquitoes under different physiological conditions.

Name of NT	Control SF_Br	Ab+_SF BR	Control SF_MG	Ab+_SF _MG	10hr PBM_Br	Ab+_10hr PBM_Br	10hr PBM_MG	Ab+_10hr PBM_MG	24hr PBM_Br	Ab+_24hr PBM_Br	24hr PBM_MG	Ab+_24hr PBM_MG
Histidine	607.77	622.52	356.52	469.70	397.58	1562.75	DC	3672.30	550.28	908.02	1968.73	DC
Serine	182.75	176.07	167.62	134.24	182.04	318.04	798.51	2636.08	197.18	350.75	1034.20	2830.31
Histamine	16.08	76.89	3.35	3.83	48.28	82.21	108.12	83.16	73.12	50.42	136.69	284.97
Aspartic Acid	272.35	96.74	115.69	24.02	209.89	143.05	1205.17	146.59	102.76	222.80	1312.08	2348.14
Glutamic Acid	604.78	255.68	513.97	397.82	523.25	425.24	DC	4530.44	486.92	690.13	DC	DC
GABA	300.01	240.89	6.38	14.50	174.40	300.76	153.31	72.01	175.86	321.81	126.79	13.94
Dopa	1.06	1.94	DC	2.00	1.45	2.04	DC	DC	2.08	1.84	DC	DC
Octopamine	5.49	0.39	BLQ	BLQ	2.29	0.20	NF	DC	0.65	1.11	NF	NF
Tyrosine	84.26	58.49	96.41	53.93	140.86	117.51	DC	DC	147.28	136.63	DC	136.63
Dopamine	1.12	0.28	0.13	0.46	1.00	0.62	69.17	26.02	1.06	0.83	11.59	25.18
Serotonin	9.33	2.25	1.92	1.35	6.06	3.39	143.62	81.14	3.59	3.72	32.70	81.37
Tyramine	0.08	BLQ	0.05	0.06	0.10	0.05	4.06	1.73	0.07	0.05	0.90	0.22
Tryptophan	21.84	68.84	11.59	16.44	36.93	74.66	1230.41	1054.85	41.40	40.70	2263.10	3650.53

* BLQ= Below Limit of Quantitation
 NF= Not Found
 DC= Detected but not calculated due to the highly suppressed Internal Standard signal
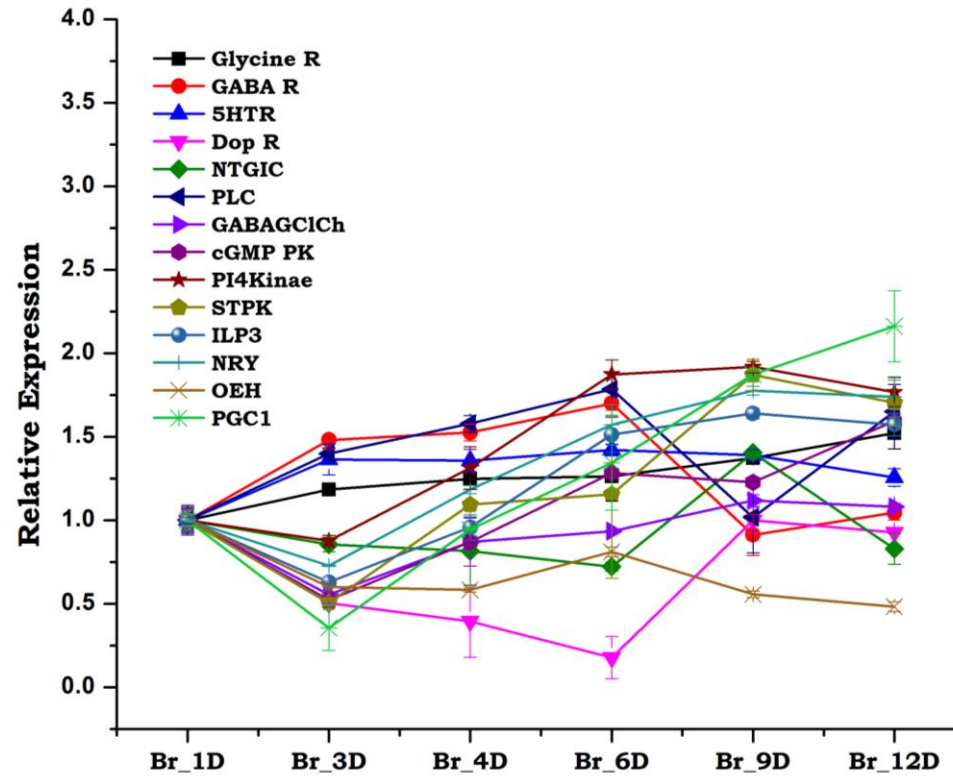
Figure S2. Differential gene expression analysis in aging non-blood fed female mosquito brain. No significant modulation of neuronal genes was observed when compared to 0-1-day old teneral mosquitoes with 12-day-old non-blood fed mosquitoes. Statistical analysis using two-way ANOVA followed by Tukey Test has implied at 0.05 level, the expression pattern of the respective genes was not statistically significant in aging mosquitoes *(n = 25, N = 3).*
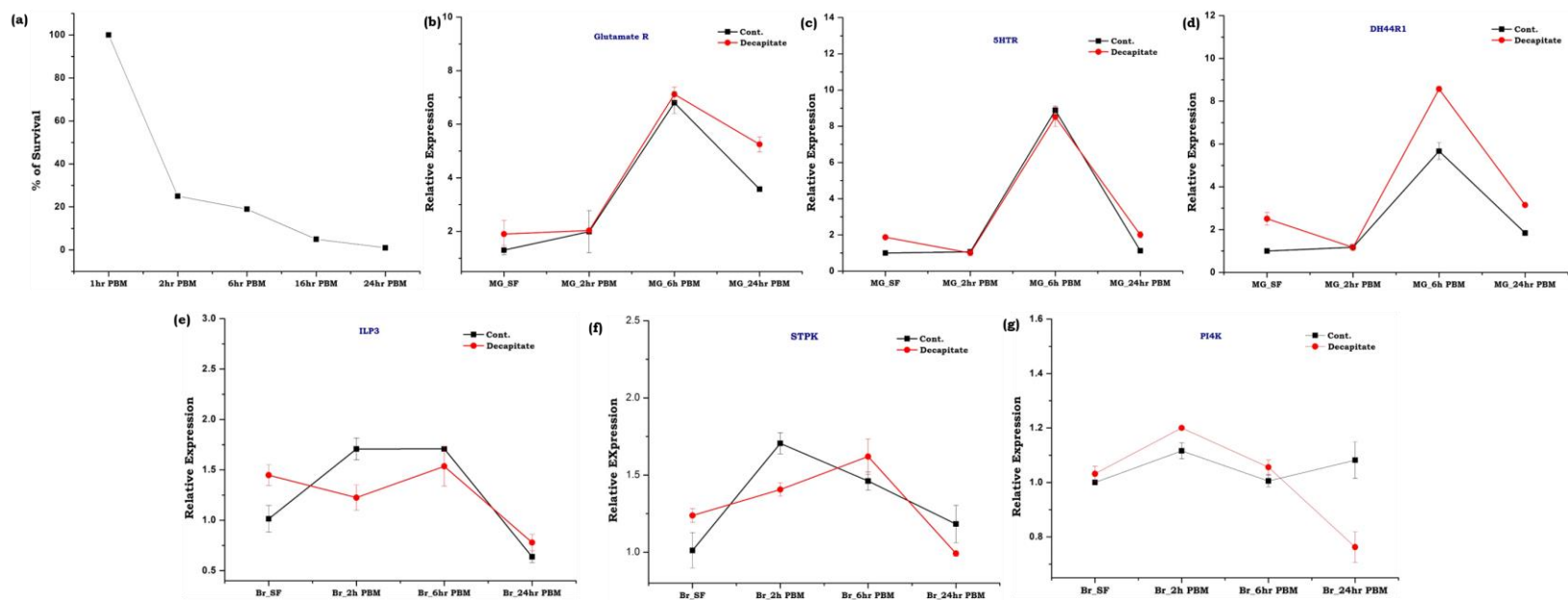
Figure S3. Transcriptional Response of neuromodulator receptor genes in naïve and decapitated blood-fed female *An. culicifacies* mosquitoes. (a) Percentage of mosquitoes that survived till 24 h after decapitation which was performed after 1h of blood-feeding. 5-6 days old sugar-fed mosquitoes were provided blood meal and mosquitoes heads were decapitated after 1 h. from the full-fed gravid females. After that, the decapitated mosquitoes were kept in a cage for recovery and count the live (mosquitoes that vibrate/move their legs or other body parts are considered as live) and dead mosquitoes (non-movable mosquitoes with visible shrinkage of the body parts at the respective time points are considered as dead) at different time points until we observed 100% mortality. The percentage of survival was calculated until 24h after blood feeding. (b-d) Relative gene expression analysis of neuromodulator receptor genes in the gut of blood-fed and decapitated female mosquitoes. (e-g) Relative gene expression analysis of neuromodulator receptor genes in the gut of blood-fed and decapitated female mosquitoes. Statistical analysis using two-way ANOVA implied that at 0.05 level the expression level the respective genes in control and decapitated females are not statistically different.
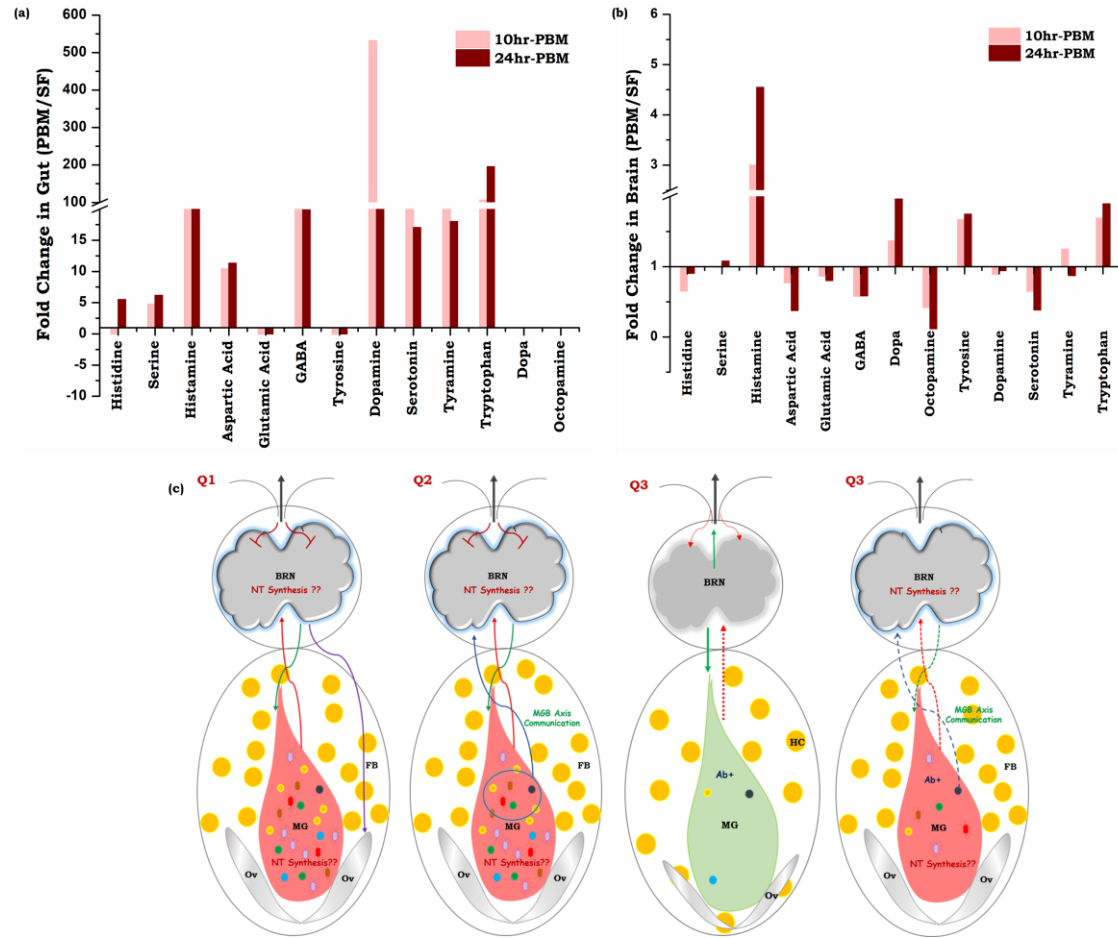
Figure S4. (a) Fold change of NT abundance in the gut of blood-fed mosquitoes. **(b)** Fold change of NT abundance in the brain of blood-fed mosquitoes. **(c)** Pictorial presentation demonstrating GBA communication in response to gut-metabolic switch in mosquitoes. Q1: Blood-feeding pauses external stimulus-guided neuro-olfactory responses, but may shift brain engagement through the vagus pathway (red arrow) to regulate actions in the distant organs such as the midgut (green arrow) and ovary (purple arrow). Here, we questioned whether increased levels of amino acids in the gut during blood meal digestion may act like an NT. Q2: Do blood-meal-induced gut flora proliferation (different colored shapes indicate diverse microbial flora) influence GBA communication in mosquitoes. Q3: Whether gut-bacterial removal by antibiotic treatment confers the establishment of microbiome-gut-brain axis (MGB) communication. BRN: Brain, MG: midgut, FB: Fat body, Ov: Ovary, Ab+: Antibiotic positive/treated.

Figure S5. Relative expression of *Agromonas* and *Rubrobacter* (gram positive bacteria) at Sugar fed and 20-24hr Post blood fed condition shows no significant change during the two-feeding status.

Figure S6. Neurotransmitter's dynamics of naïve and aseptic mosquitoes collected from sugar fed and blood fed conditions.

Figure S7. Molecular catalog of brain-specific immune transcripts. (a) Molecular catalog of the different classes of immune genes expressed in brain tissue; (b) Differential expression patterns of the brain immunome as determined by the number of sequences that appeared in each RNA-Seq data of naïve and blood-fed mosquito brains.

Representative UHPLC-MS/SRM chromatogram of Blank:

Representative UHPLC-MS/SRM chromatogram of Standard:

RT: 7.28 A: 1,1372 — NL: 3.70E2 326.11 → 110.00 Histidine
RT: 7.25 A: 6966 — NL: 2.38E2 329.00 → 113.00 Histidine D3
RT: 8.26 A: 280710 — NL: 1.23E4 276.10 → 171.04 Serine
RT: 8.23 A: 190982 — NL: 7.82E3 279.10 → 171.00 Serine D3
RT: 9.31 A: 70529 — NL: 1.75E3 282.37 → 138.00 Histamine
RT: 9.28 A: 48330 — NL: 1.10E3 286.37 → 142.00 Histamine D4
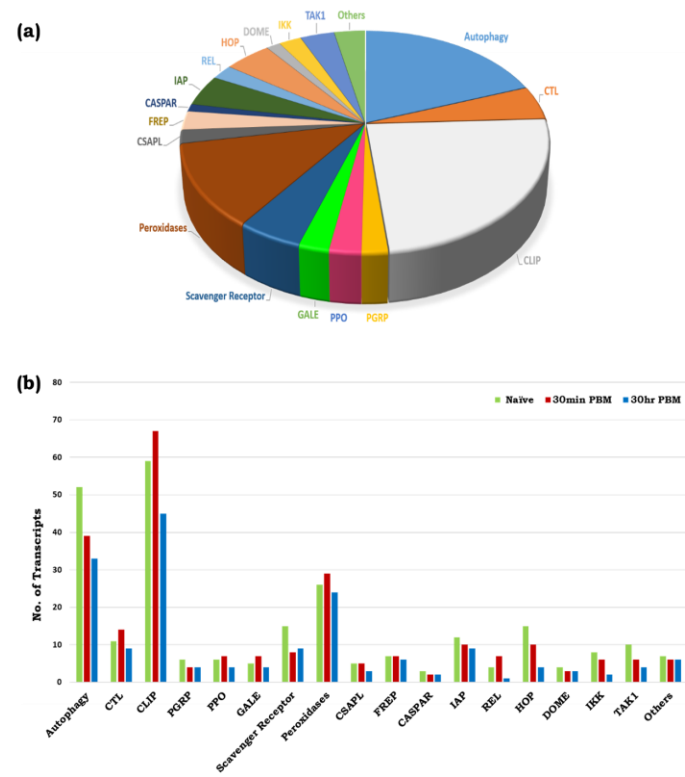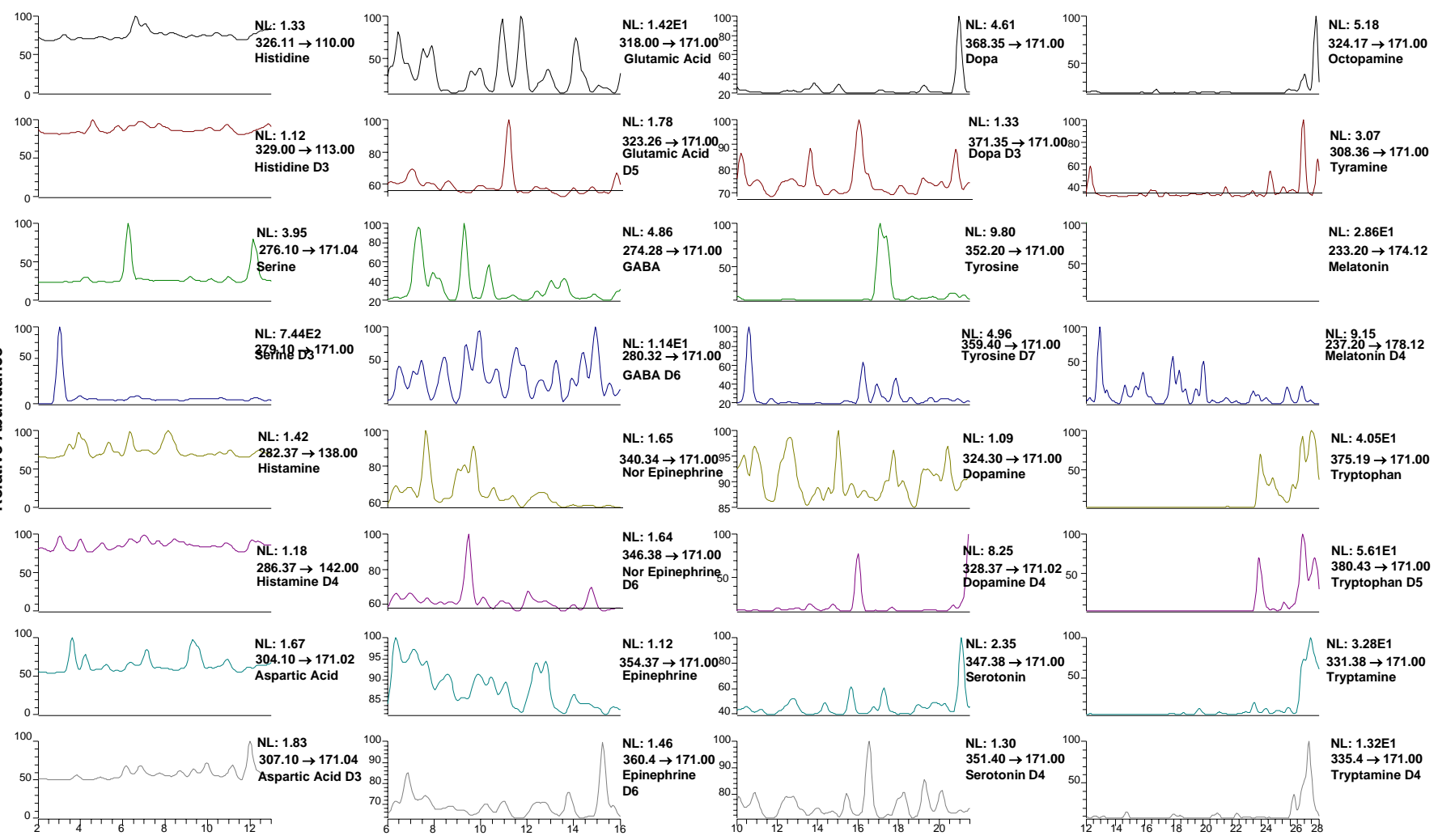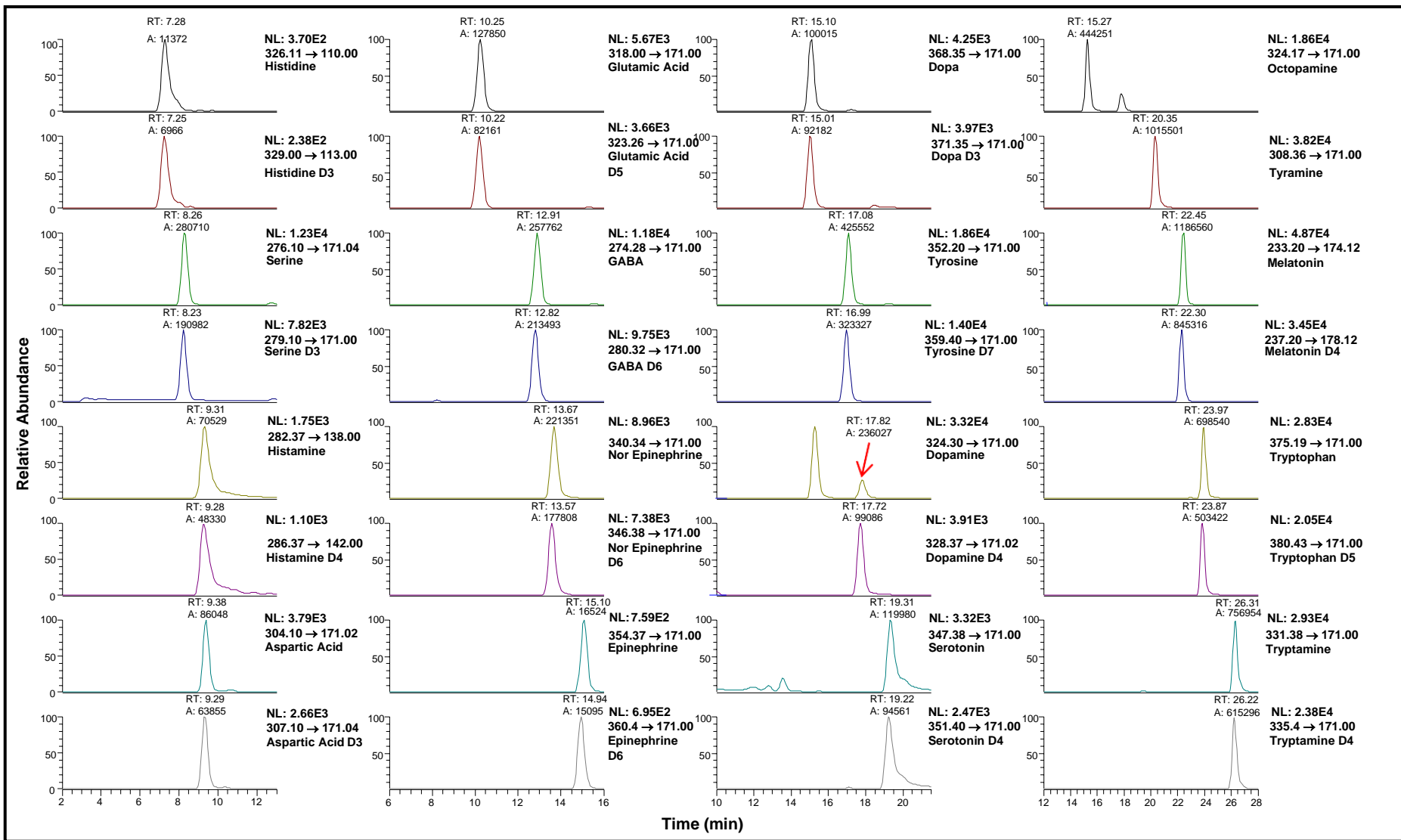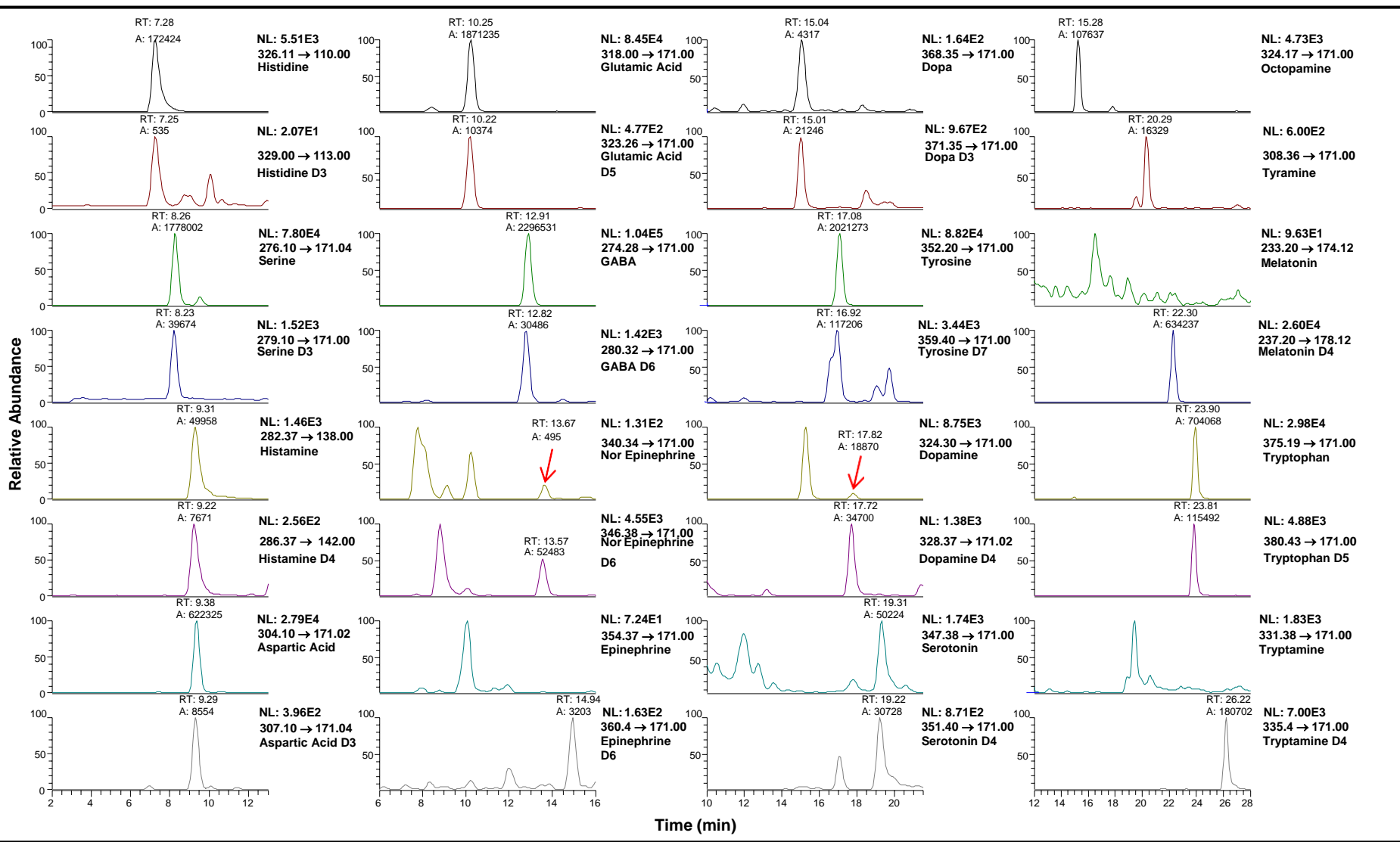RT: 9.38 A: 86048 — NL: 3.79E3 304.10 → 171.02 Aspartic Acid
RT: 9.29 A: 63855 — NL: 2.66E3 307.10 → 171.04 Aspartic Acid D3

RT: 10.25 A: 127850 — NL: 5.67E3 318.00 → 171.00 Glutamic Acid
RT: 10.22 A: 82161 — NL: 3.66E3 323.26 → 171.00 Glutamic Acid D5
RT: 12.91 A: 257762 — NL: 1.18E4 274.28 → 171.00 GABA
RT: 12.82 A: 213493 — NL: 9.75E3 280.32 → 171.00 GABA D6
RT: 13.67 A: 221351 — NL: 8.96E3 340.34 → 171.00 Nor Epinephrine
RT: 13.57 A: 177808 — NL: 7.38E3 346.38 → 171.00 Nor Epinephrine D6
RT: 15.10 A: 16524 — NL: 7.59E2 354.37 → 171.00 Epinephrine
RT: 14.94 A: 15095 — NL: 6.95E2 360.4 → 171.00 Epinephrine D6

RT: 15.10 A: 100015 — NL: 4.25E3 368.35 → 171.00 Dopa
RT: 15.01 A: 92182 — NL: 3.97E3 371.35 → 171.00 Dopa D3
RT: 17.08 A: 425552 — NL: 1.86E4 352.20 → 171.00 Tyrosine
RT: 16.99 A: 323327 — NL: 1.40E4 359.40 → 171.00 Tyrosine D7
RT: 17.82 A: 236027 — NL: 3.32E4 324.30 → 171.00 Dopamine
RT: 17.72 A: 99086 — NL: 3.91E3 328.37 → 171.02 Dopamine D4
RT: 19.31 A: 119980 — NL: 3.32E3 347.38 → 171.00 Serotonin
RT: 19.22 A: 94561 — NL: 2.47E3 351.40 → 171.00 Serotonin D4

RT: 15.27 A: 444251 — NL: 1.86E4 324.17 → 171.00 Octopamine
RT: 20.35 A: 1015501 — NL: 3.82E4 308.36 → 171.00 Tyramine
RT: 22.45 A: 1186560 — NL: 4.87E4 233.20 → 174.12 Melatonin
RT: 22.30 A: 845316 — NL: 3.45E4 237.20 → 178.12 Melatonin D4
RT: 23.97 A: 698540 — NL: 2.83E4 375.19 → 171.00 Tryptophan
RT: 23.87 A: 503422 — NL: 2.05E4 380.43 → 171.00 Tryptophan D5
RT: 26.31 A: 756954 — NL: 2.93E4 331.38 → 171.00 Tryptamine
RT: 26.22 A: 615296 — NL: 2.38E4 335.4 → 171.00 Tryptamine D4

Relative Abundance

Time (min)

Representative UHPLC-MS/SRM chromatogram of sample Ac_SF_Br:

Representative UHPLC-MS/SRM chromatogram of sample Ac_SF_MG: