Microbiome-Gut-Brain-Axis communication regulates metabolic switch in the mosquito *Anopheles culicifacies*

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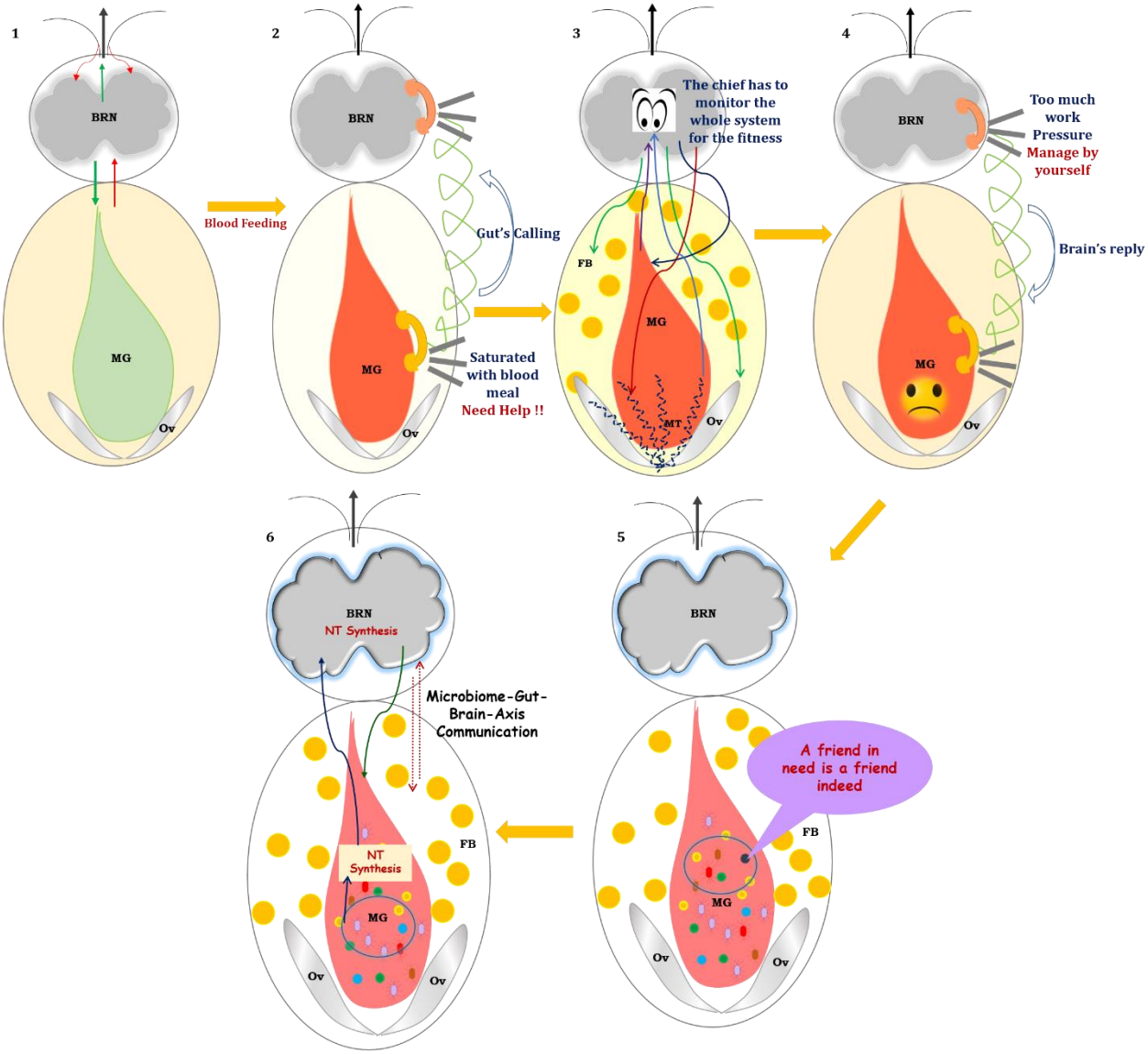
Abstract:

A well coordinated neuro-olfactory regulation is essential for a successful host seeking and blood feeding behavior of adult female mosquitoes. Although, the crucial role of mosquito tiny brain during host selection/choice is of prime interest but how blood meal uptake modulate brain functions which consequently empower the cognition in mosquitoes remains unclear. Here we report that blood meal induced gut 'metabolic switch' shift brain function from external communication to inter-organ management. An exclusive induction of oxidation reduction associated proteins and enhancement of brain's energy metabolism indicate the active functioning of the brain probably to maintain physiological homeostasis. A spatial and temporal change in the expression pattern of neuro-signaling molecules and other neuro-modulators in the brain designate that a guided neuro-stimulation and endocrine regulation is enough to manage the brain-distant organ communication. Analogous to brain, significant modulation of the neuro-modulator receptor genes in the gut signifies its function as 'Second Brain' during metabolic switch in mosquitoes. Furthermore, quantitative analysis of neuro-transmitters (NTs)revealed that the gut of mosquitoes is one of the potent source of NTs during altered metabolic conditions. Finally, quantitative data on NTs dynamics of naïve and aseptic mosquitoes establish the concept of microbiome-gut-brain-axis communication in adult female mosquitoes.

Graphical abstract:



Introduction:

Central nervous system i.e. the brain is a privileged organ in shaping animal's behavior from lower to higher taxa of which insect's tiny brain is famous for performing amazingly sophisticated behavior. Thus, insects are either enough smart to squeeze more information from limited number of neurons or they use some different algorithms to process information in their microscopic brain system [1]. In particular to mosquitoes, while navigating towards a vertebrate host for blood feeding the brain engagement of adult female mosquitoes is more complex than any other insect species [2–5]. A crucial interaction of olfactory derived odorant binding proteins (OBPs) and olfactory receptors (Ors) with the environmental guided chemical cues may have direct impact on mosquito's behavioural properties [6,7]. But how this chemical information influences decision

making abilities of the miniature brain of mosquitoes is yet unknown which could reveal out of the box technique to disrupt mosquito host-seeking and blood feeding behavior.

Upon locating a suitable vertebrate host, a positive feeding decision directs salivary glands to facilitate blood meal uptake in less than 2 minutes. Fast engorgement of mosquito's gut with blood meal causes a drastic change of innate physiological status from sugar to protein rich blood, which results gut 'metabolic switch' activity. This metabolic transition boosts multiple organs engagement such as malpighian tubules for osmoregulation, gut for biochemical activity together with progressive blood meal digestion, fat body for nutrient mobilization and initiation of vitellogenesis and ovary for egg maturation [8–12]. Thus a rapid alteration of physiological homeostasis also creates an increased burden on the central nervous system to manage inter-organ communication. Moreover, according to the previous concept of metabolic switch, changes in the cellular and molecular activity of neuronal circuits not only facilitate inter-organ communication but also improve neural plasticity and cognition [13,14]. In fruitfly, several neuromodulators such as neuropeptides, neurotransmitters and neurohormones have been demonstrated to play crucial role in neurosynaptic signal transmission and inter-organ communication [12,15]. But, how mosquitoes brain orchestrate gut metabolic switch activities has not been addressed yet. Only few recent genetic studies indicate the significant role of some neuropeptides (Neuropeptide-Y, Short-neuropeptide F and Allatostatin-A) and its receptors in the supression of host-seeking and paternity enforcement in *Aedes aegypti* mosquitoes [16–18]. Furthermore, emerging knowledge highlights that there is an immence cross-talk between the gut and the brain which is predominantly mediated by the enteric nervous system (vagus nerve) and thus referred as 'Second Brain' [19]. Nowadays, it is also dicernable that the bi-directional communication between the gut-brain is influenced by gut-endosymbionts. And the nexus of communication among microbiota-gut-vagus-brain axis is crucial for maintaining metabolic homeostasis, mood and perception. [20–22]. Though, blood meal induced gut-flora proliferation has been well demonstated [23] but their neuromodualtory functions in mosquitoes remains elusive.

We recently demonstrated that a synergistic action of olfactory encoded OBPs/Ors drives blood feeding associated complex behavioral responses. Next, to track spatial and temporal changes in the downstream neuro-modulatory pathways, we performed RNA-Seq analysis of brain tissue collected from naïve and blood fed *An. culicifacies* mosquitoes which is a dominant malarial vector in rural India. Here we provide an evidence that how (i) gradual changes in the brain transcripts and enhanced energy metabolism manages metabolic switch activities (ii) gut metabolic switch establish gut-brain-axis communication; and (ii) blood meal induced gut flora expansion affects neurotransmitter dynamics in the mosquito's brain. Our findings establish a conceptual understanding of bi-directional gut-brain-axis communication and gut microbial impact in the mosquitoes.

Material and methods

Fig. S1 represents a technical overview of the current investigation.

Mosquito rearing and maintenance: A cyclic colony of *An. culicifacies* mosquito, sibling species A was reared and maintained at 28±2°C temperature and relative humidity of 80% in the central insectary facility at ICMR-National Institute of Malaria Research [24,25]. For routine rearing adult female mosquitoes were fed on rabbit. All protocols for rearing and maintenance of the mosquito culture were approved by ethical committee of the institute.

RNA isolation and Transcriptome Sequencing Analysis: For RNA-Seq study, the brain tissues were dissected from 0-1-day old, 30 min post blood fed and 30 hrs post blood fed cold anesthetized *An. culicifacies* mosquitoes and collected in Trizol reagent by decapitation of the heads followed by application of gentle pressure over the head to pull out the brain tissue from the head cuticle. Then, total RNA was extracted from the collected brain tissues (approximately 30 mosquitoes was pooled to form one single sample) and a double-stranded cDNA library for each set of naïve, 30min and 30h post blood fed, were prepared by a prior well-established PCR-based protocol [24,26]. For transcriptome sequencing, the Illumina MiSeq 2 X 150 paired-end library preparation protocol was followed. The bioinformatics data analysis of pipeline is shown in Fig S1. Briefly, raw reads from each set was processed for removing the adaptors and low quality bases (<20). A de-novo clustering was used to build final contigs/transcripts dataset using CLC Genomics workbench (V6.2) (31) with default parameters (contig length ≥ 200, Automatic word size: Yes, Perform Scaffolding: Yes, Mismatch cost: 2, Inserstion cost: 3, Deletion cost: 3, length fraction: 0.5, Similarity fraction: 0.8). Finally, assembled transcriptome were used for CDS prediction and annotation using transdecoder and BLASTX at e-value $1e^{-6}$ respectively. For a comprehensive differential gene expression analysis, we used exactly the same protocol as mentioned previously [6,24]. Additionally, to identify the differentially expressed gene, associated with certain biological and molecular processes we performed gene-list enrichment analysis using Kobas 3.0 web server. The unique appearance of certain pathways in different brain samples were screened depending on the p-value (<0.5).

PCR based gene expression Analysis: To establish the concept of metabolic switch and inter-organ communication in mosquitoes we targeted *An. culicifacies* brain, midgut, malphigian tubule and ovary tissues. The respective tissues were dissected and collected from both naïve sugar fed and blood fed mosquitoes at different time points. At first, the tissues were collected from 5-6 day old 25-30 naïve sugar-fed adult female mosquitoes. Next, adult female mosquitoes of the same cohort were offered blood meal by offering a live animal (rabbit) and the desired tissues were collected after 2hrs of blood feeding. Later, the full blood-fed mosquitoes were separated and kept in a proper insectary condition and the tissues were collected at the following time point of post blood feeding (PBM): 2hr PBM, 8-10hr PBM, 24-30hr PBM, 48hr PBM, 72hr PBM for tissue specific details expression analysis of the respective genes. The different tissues were pooled accordingly in Trizol and total RNA was extracted followed by cDNA preparation. Differential gene expression analysis was performed using the normal RT-PCR and agarose gel electrophoresis protocol and was statistically analyzed using student ‘t’ test. For relative gene expression analysis,

SYBR green qPCR master mix and Biorad CFX 96 Real-Time PCR machine were used. PCR cycle parameters involved an initial denaturation at 95°C for 5 min, 40 cycles of 10 s at 95°C, 15 s at 52°C, and 22 s at 72°C. Fluorescence readings were taken at 72°C after each cycle. The final steps of PCR at 95°C for 15 secs followed by 55°C for 15 secs and again 95°C for 15 secs were completed before deriving a melting curve. Each experiment was performed in three independent biological replicates for better evaluation of relative expression. Actin or Rps7 gene were used as internal control in all the experiment and the relative quantification was analysed by $2^{-\Delta\Delta Ct}$ method [27].

ROS determination assay of blood fed mosquitoes brain: To unravel the origin of oxidative stress response in blood fed brain we performed Reactive oxygen species (ROS) determination assay by incubating the brain tissue dissected from naïve and blood fed mosquitoes with a 2 mM solution of the oxidant-sensitive fluorophores, CM-H2DCFDA [5-(and-6)-chloromethyl-29,79-dichloro-dihydrofluorescein diacetate, acetyl ester] (Sigma). After a 20-min incubation at room temperature in the dark, the brain tissues were washed thrice with PBS and then transferred to a glass slide in a drop of PBS and checked the fluorescence intensity at wavelength 490 nm under a fluorescent microscope.

Antibiotic treatment of mosquitoes: To establish the concept of microbiome-gut-brain-axis communication we disrupt the gut-commensal bacteria through antibiotic treatment. For the removal of gut bacteria, the pupae were emerged in a washed and aseptic mosquito cage made up of muslin cloth. Antibiotic diet was provided to the mosquitoes, newly emerged from pupae for 4-5days by mixing 10% sucrose solution with 10 µg of penicillin-streptomycin/ml and 15 µg gentamicin sulphate in it. To avoid any contamination, the antibiotic regimen was changed on daily basis. After 4-5days of antibiotic treatment, mosquitoes were provided blood meal through rabbit.

Sample processing and MS analysis for neurotransmitter quantification: For absolute quantification of neurotransmitters, mosquitoes were decapitated and brains were pull out from the head cuticle and quickly collected in an Eppendorf containing 50µl of 1% ascorbic acid and immediately freeze it. For each set ~60-65 mosquitoes brain or gut were pooled in a single tube. All samples were stored at -80 °C until further used. Each sample was extracted with 3X volume of extraction solvent. Samples were vortexed and refrigerated for 10 to 15 minutes at 4 °C. Samples were then subjected to sonication in a bath-type ultra-sonicator in pulses (twice, for 1 min each). Samples were then centrifuged at 14500 rpm for 5 min at 4 °C. Supernatants were separated and dried under vacuum. Dried samples were spiked with internal standards (ISTDs) and derivatized, cleaned-up and prepared for LC-MS injections as per protocol described earlier [28].

Briefly, Standards (STDs) were spiked in 200µl of extraction solvent (acidic acetone (0.1% FA) containing 0.5mM ascorbic acid) and dried under vacuum. ISTDs were spiked to dried STDs, followed by addition of 80µL borate buffer (200mM, pH 8.8) containing 1mM ascorbic acid. To the above mixture 10µl of 0.1 N NaOH was added followed by addition of AQC (from 1 mg mL−1 stock). Samples were incubated at 55°C for 10 min. The reaction was stopped by addition of 500µL of acidic water (0.1% FA). The derivatized standards were cleaned-up using RP-SPE cartridges using the previously optimized protocol [29][28]: activation with methanol, equilibration with

water (0.1% FA), loading of samples, washing (twice) with water (0.1% FA) and elution with acetonitrile: methanol (80:20) containing 2% FA. The eluate was dried under vacuum and reconstituted in 50µL of 0.5% acetonitrile. 10µL of reconstituted standards were injected for UHPLC-MS/SRM analysis.

Data was acquired on a TSQ Vantage (triple stage quadrupole) mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) coupled with an Agilent 1290 Infinity series UHPLC system (Agilent Technologies India Pvt. Ltd.). The UHPLC system is equipped with a column oven (set at 40 °C) and a thermo-controller for maintaining the auto-sampler at 10 °C. A C-18 column (2.1 × 100 mm, 1.8µm, Agilent, Inc.) was used to perform the separation. The mobile phase Solvent A was 10mM ammonium acetate in water containing 0.1% formic acid and Solvent B was acetonitrile containing 0.1% formic acid. The gradient was optimized to get maximum separation (2% B at 0 min, 2% B at 3 min, 20% B at 20 min, 35% B at 25 min, 80% B at 25–27 min, 2% B at 27–35 min) at a flow rate of 200µL min−1. Operating conditions were as follows- ionization mode: positive; spray voltage: 3700 V; capillary temperature: 270 °C; source temperature: 80 °C; sheath gas: 30 (arbitrary units); auxiliary gas: 10 (arbitrary units); collision gas: argon. Parent and product masses, S-lens voltages and collision energies were used as per the previously optimized method [28][29].

Results & Discussion

Hypothesis: Since, neuro system is a versatile centre of chemical information exchange, we hypothesize a minor change in the innate physiological status may have strong impact on mosquito's day to day life. Importantly blood meal uptake causes a drastic metabolic changes, which we named "gut metabolic switch", engages multiple organs simultaneously. We hypothesize that upon blood feeding mosquitoes' brain functions may shift from external communication to internal management such as (a) initiation of diuresis b) finding a resting site for digestion of blood meal in the midgut; (c) distribution of amino acids, generated through the degradation of protein rich blood meal; (d) active engagement of fat body and ovary for egg maturation for life cycle maintenance. We propose and decode the molecular basis that how mosquito brain (i) coordinate pre and post blood meal associated metabolic switch activities and (ii) establish a functional correlation with distant organs during first ~30 hrs of blood meal digestion process in the gut (Fig. 1).

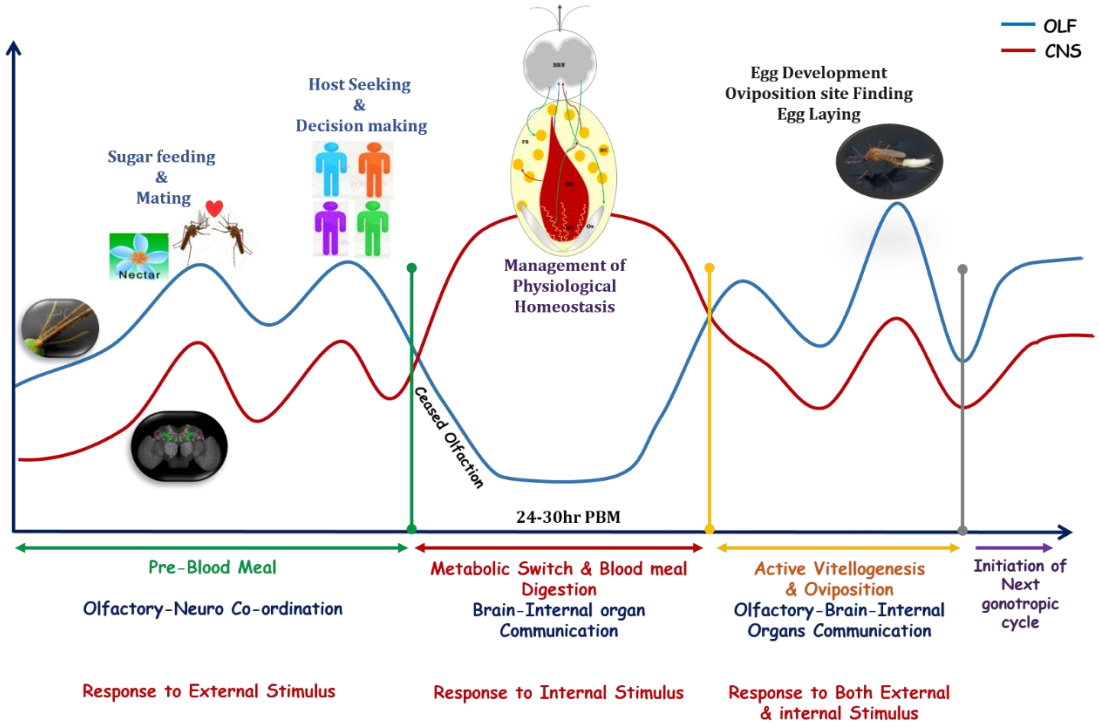


Fig. 1: <u>Proposed working hypothesis to establish the functional co-relation between gut metabolic switch and brain functions in adult female mosquitoes:</u> Behavior of any organism is a very complex events which needs a tight coordination between sensory system and neuronal system. After emergence from pupae the dynamic and co-ordinated change in the neuro-olfactory system regulate different behavioral activities such as mating, sugar feeding, vertebrate host-seeking etc. This pre-blood meal associated behaviors are guided by external stimulus followed by neuronal decision making. Whereas once the female mosquitoes take blood meal, their olfactory responses are ceased out which consequently minimize brain and environmental (external) communication. But blood feeding causes a global change in the physiological homeostasis and multiple tissues (midgut, malphigian tubule, ovary, fat body) are engaged to manage the systemic equilibrium. Here we hypothesize that metabolic switch mediated internal stimulus may accelerate brain functions possibly to supervise other tissues to perform their respective functions at least for first 30hrs till blood meal digestion completed in the gut. After 30-40hrs of blood feeding reactivation of olfactory system occurs which again establish olfactory-neuro co-ordination to perform next level of behavioral activities such as oviposition and initiation of second gonotropic cycle. Blue and red line indicates possible functional pattern of the olfactory system and the brain respectively.

Blood meal trigger brain engagement by inducing energy metabolic activities: A comparative RNAseq analysis of 1-2 days old naïve, 30min and 30 hrs. post blood fed mosquito's brain showed a gradual suppression of brain specific proteins (Fig. 2a), except exclusive induction of oxidation-reduction process associated proteins (Fig. 2b). We interpreted that a rapid heme detoxification in midgut may have resulted in the Reactive Oxygen Species (ROS) mediated stress response in the blood fed brain. We, however, failed to detect any signal of oxidative stress in 2mM solution of the oxidant-sensitive fluorophores, CM-H2DCFDA [5-(and-6)-chloromethyl-29,79-dichloro-dihydrofluorescein diacetate, acetyl ester] (data not shown). An in depth analysis of oxidation-reduction category transcripts showed an enrichment of mitochondrial activity proteins such as 2-oxoglutarate dehydrogenase, NADH dehydrogenase, glutathione peroxidase etc. A comparative pathway prediction analysis further showed an exclusive induction of several unique pathways linked to (a) energy metabolism; (b) neurotransmitter synthesis; (c) neurite outgrowth and synaptic

transmission (Fig. 2c). Together these data indicated that blood meal associated gut metabolic switch may trigger a "hyper energy" state of mosquito brain.

To verify the above presumption, we profiled the expression pattern of PGC-1 gene (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha), an important transcriptional co-activator which regulate genes involved in energy metabolism [30][31][32]. A persistent elevation of PGC-1 ($P \leq 0.009$ at 8hr PBM, 0.007 at 30hr PBM), and a parallel enrichment of glycolysis and TCA cycle gene pyruvate kinase ($P \leq 0.0176$) and oxoglutarate dehydrogenase ($P \leq 0.0019$) respectively, indicated an enhanced mitochondrial activity in the brain of blood fed mosquitoes. (Fig. 2d, e). Next we tested whether the amino acids generated through blood meal digestion, or the trehalose, a non-reducing disaccharide act as raw material for brain's energy metabolism. Although, trehalose is the primary energy source in the insects' brain [33][34], but we observed a sequential increment of amino acid ($P \leq 0.0515$) as well as trehalose transporter ($P \leq 0.0071$) genes in the blood fed brain (Fig. 2f). Together these data indicated that both amino acids and trehalose, may synergistically communicate the nutritional signal to brain via gut-hemolymph, for active management of multi-organ communication (Fig. 2g).

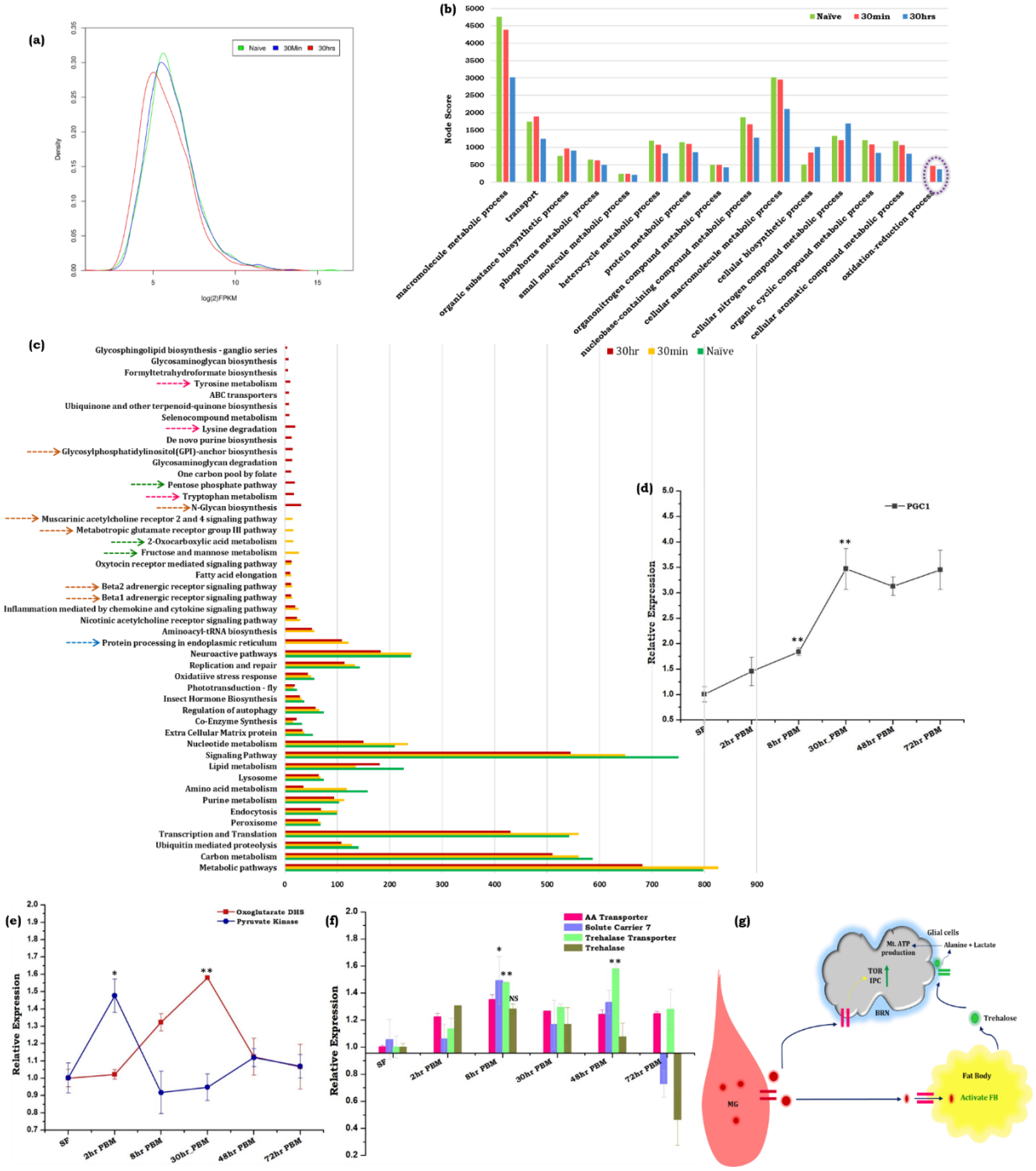


Fig. 2: <u>Blood meal cause notable changes in the molecular architecture of the brain tissue</u>: (a) Comparison of read density map of the Naive; 30Min and 30hrs post blood meal (PBM) transcriptomic data of brain tissue; **(b)** Functional annotation and molecular cataloguing of brain transcriptom**e** (Biological Process/Level4/Node score); Purple circle highlighted the unique category of genes that appeared in the brain tissue after blood meal intake; **(c)** KOBAS 3.0 software mediated gene list enrichment analysis of naïve and blood fed brain tissues and comparative pathway map of the same. Green arrow linked to energy metabolic pathways, pink arrow linked to neurotransmitter synthesis pathway and brown arrow indicates neurite out-growth and synaptic transmission; **(d)** Relative expression profiling of PGC-1 gene in the brain of naïve and blood fed mosquitoes. At first the brain tissue was collected from naïve sugar fed mosquitoes. Then mosquitoes were provided blood meal followed by brain tissues collection at different time points after blood feeding, viz. 2hr-PBM (Post-Blood-Meal); 8hr-PBM; 30hr-PBM; 48hr-PBM; 72hr-PBM; **(e)** Transcriptional profiling of transcripts related to energy metabolism in the brain tissue of naïve and blood

fed mosquitoes at different time points; **(f)** Comparative transcriptional response of amino acid transporters and trehalose transporter along with trehalase enzyme in the brain tissue after metabolic switch; **(g)** Proposed possible mode of signal flow from midgut (MG) and fat body (FB) towards brain after metabolic switch. Amino acids (red circle) generated in the MG upon blood meal digestion are exported in the hemolymph which then send the nutritional signal towards brain either through direct transport or indirectly through FB. After amino acid mediated activation of FB, it may synthesize and secrete trehalose into the hemolymph as a response of nutrient rich condition which then transport towards brain. Trehalose enters into the brain through trehalose transporter residing in the surrounding glial cells and metabolized to alanine and lactate which then transported into the higher brain and utilized for ATP production.

Spatial and temporal modulations of neuro-signaling regulate metabolic switch activities:

The above data reinforce us to cogitate that how brain's energy consumption is associated with its administrative function for the regulation of gut metabolic switch activities. In naive sugar fed mosquitoes, brain energy consumption is optimal to drive routine behavioral events like flight, mating and host seeking, which are dominantly managed by the external environmental stimuli. Here, we hypothesize that blood meal uptake may temporarily pause the external communication, but boost a 'hyper energy' state, possibly to shift brain engagement for the management of global physiological homeostasis. Since, no knowledge available that how metabolic switch modulate brain function, first we targeted the transcripts required for the key events of synaptic signal transmission (Fig. 3a). Further, to understand the switching mechanism (switch off external stimuli and switch on internal stimuli) of brain, we evaluated blood meal associated transcriptional response of selected transcripts regulating either receptor-mediated neuronal signalling or cellular signalling process during synaptic transmission [35].

The obtained result on neurotransmitter and biogenic amine receptor genes such as serotonin receptor, dopamine receptor, octopamine receptor, GABA receptor etc. showed a limited change in response to blood meal. This may be a possible mechanism to arrest brain's response against external stimuli, and prevent the over-excitation of the neurons (Fig. 3b). Due course of that, the cellular signal transduction proteins such as cGMP protein kinase, phospholipase C, GABA gated chloride channel, serine threonine protein kinase, exhibited a significant modulation in response to metabolic switch (Fig. 3c). These findings strongly suggested that rapid blood meal may drive and switch brain engagement from 'external stimulus to internal stimulus' of acute metabolic changes which could be a possible strategy of brain to manage blood meal associated complex events ongoing in distant organs (Fig. 3d).

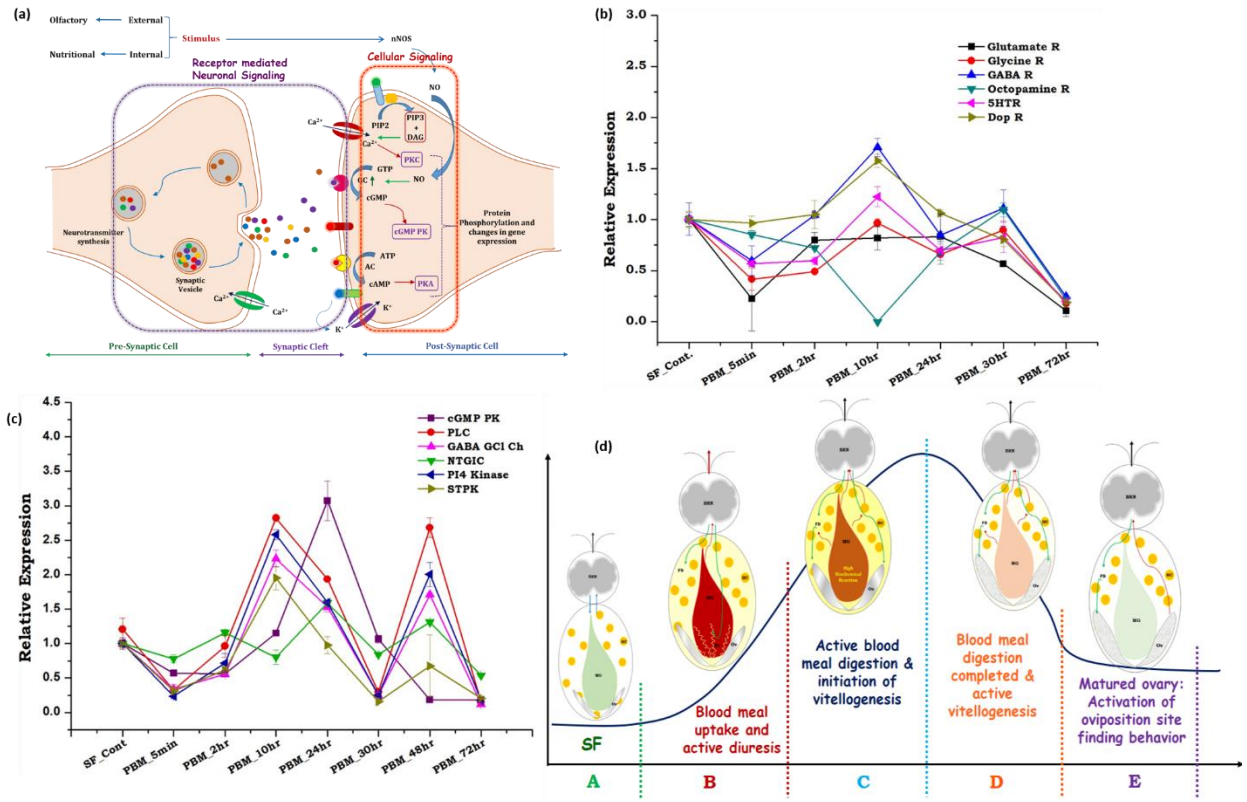


Fig. 3: <u>Metabolic switch induced modulation of neuro-signalling and inter-organ communication</u>: (a) Synaptic signal transmission and probable mechanism of neuro-signalling. After activation by external and/or internal stimulus the synaptic vesicle of the presynaptic neuron, containing the neurotransmitters are released into the synaptic cleft. Binding of the respective neurotransmitter with their cognate receptors activate the downstream signal transduction process in the postsynaptic neuron and thus activate and transmit the initial signal through the interconnecting neurons; **(b)** Transcriptional response of neurotransmitter receptor genes according to the detail blood meal time series experiment. Brain tissues were collected from 5-6day old naïve sugar fed adult female mosquitoes. Then mosquitoes were provided blood meal and the brain tissues were collected at different time point after blood feeding viz. 5min post blood meal (PBM-5min), PBM-2hr, PBM-10hr, PBM-24hr, PBM 30hr and PBM-72hr. Glutamate R: Glutamate Receptor; Glycine R: Glycine Receptor; GABA R: Gamma-Aminobutyric Acid Receptor; Octopamine R: Octopamine Receptor; 5HTR: Serotonin Receptor; Dop R: Dopamine Receptor; **(c)** Relative expression profiling of the genes involved in signal transduction molecules according to the detail blood meal time series experiment. Brain tissues were collected from 5-6day old naïve sugar fed adult female mosquitoes. Then mosquitoes were provided blood meal and the brain tissues were collected at different time point after blood feeding as described above. cGMP PK: Cyclic GMP Protein Kinase; PLC: Phospholipase C; GABA GCl Ch: GABA Gated Chloride Channel; NTGIC: Neurotransmitter Gated Ion Channel; PI4 Kinase: Phosphatidyl-inositol-4-Kinase; STPK: Serine Threonine Protein Kinase; **(d)** Representation of brain function during inter-organ communication after metabolic switch. Brain actions are subdivided into different phases. In sugar fed status brain manage daily behavioral activities but as soon as mosquitoes take blood meal, hyper-activation of brain functions occurs to manage multiple physiological events such as diuresis process for osmotic regulation, active blood meal digestion in the midgut, co-ordinate with MG, FB and ovary for egg maturation and vitellogenesis. After 30-40hrs brain function comes to normal level and olfactory reactivation occurred.

Tissue specific modulation of neuromodulators/receptors mediates inter-organ communication: In order to validate the above presumption of brain-inter-organ communication, we monitored the temporal and spatial expression of 21 key genes (Table 1) having blood meal associated function in their targeted tissue such as midgut (MG), ovary (Ov), and Malpigian tubules (MT). Notably, we observed a significant upregulation of ILP3, and a time dependent modulation of other neuropeptide (Neuropeptide Y, Leukokinin) and neuro-hormones (OEH, DH44 and ARMAA) in the blood fed mosquitoes brain (Fig. 4a, b, c). We interpreted that a gradual induction of ILP3 synthesis and OEH secretion from brain's neurosecretory cells, may activate the ovaries for the synthesis of ecdysteroids to initiate vitellogenesis process [36][37][38]. A transient increase of NRY immediately after blood feeding, may be due to gut distension, but a significant increase after 24hrs and 72hrs may negatively regulate host-seeking behavioral activities, which also supports previous findings [39].

Next, we asked that how the dynamic change of the neuromodulators in blood fed brain influence distant organs response such as diuresis regulation by malpighian tubule, blood digestion process in midgut, oocyte maturation in ovary. Transcriptional profiling of selected neuropeptide, neurotransmitter receptor transcripts (Table 1) indicated that blood meal triggers an immediate and long-lasting (~48hrs PBF) impact on the gut-neuro transcripts expression (Fig. 4d). A parallel observation of an early induction (2hrs PBF) of serine threonine protein kinase (MAPK activated protein kinase), and late expression of Akt kinase (48hrs PBF) in the ovary suggested a controlled regulation of nutritional signalling pathway to favor vitellogenesis process (Fig. 4e). Likewise, a unique pattern of diuretic hormone (8hr PBF) and potassium dependent sodium calcium exchanger gene (24h PBF) expression in the Malpighian tubule suggested an active diuresis process till 24hrs post blood meal (Fig. 4f).

Table 1: Details of the selected transcripts used to understand inter-organ communication during metabolic switch event.

Sl. No.	Gene Name	Synthesized From	Target Tissue	Possible Function	Target Tissue for Expression study
1.	ILP1	MNSC of brain	Ovary	Halt ovarian maturation [40]	Brain, midgut
2.	ILP3	MNSC of brain	Midgut, Ovary, Fat Body, Hemocyte	Nutrient storage by FB, regulation of digestive enzymes by MG, Ecdysteroid production from ovaries, immune response by HC [37][11][41]	Brain, midgut
3.	Leucokinin	Abdominal ganglia	Gut, Malphigian tubule	Regulation of fluid secretion, ionic balance [42]	Brain
4.	PTTH - Prothoracicotropic Hormone	Brain	Not Known	Diapause and blood feeding [43]	Brain

5.	Neuropeptide Y-NRY	NSC of brain	Brain	Host-seeking inhibition [18][39]	Brain
6.	Leucokinin Receptor	Multiple tissues	Multiple tissues	Regulation of fluid secretion, ionic balance [44]v	Brain, midgut
7.	Diuretic hormone 44 (DH44)	Gut endocrine cells	Malphigian tubule	Regulation of diuresis [45]	Brain, midgut
8.	OEH - Ovary Ecdysteroidogenic Hormone	MNSC and ventricular ganglia of brain	Ovary	Induces ecdysone production from ovary after blood feeding [44]	Brain
9.	ARMAA - Aromatic-L-amino-acid decarboxylase	Multiple tissues	Multiple tissues	Synthesis of serotonin neurotransmitter and regulation of multiple physiological processes	Brain
10.	DH44R1	Malphigian tubule	Malphigian tubule	Regulation of Diuresis [45][44]	Midgut and malphigian tubule
11.	CCHamide Receptor 2	CCHamide2 snthesized from gut endocrine cells	Multiple tissues	Nutrient dependent regulation of ILPs from brain [44]	Midgut
12.	5HTR - Serotonin Receptor	Multiple tissues	Multiple tissues	Multiple behavioural and physiological processes [46][47]	Midgut
13.	Glutamate R - Glutamate Receptor	Multiple tissues	Multiple tissues	Olfactory ionotropic glutamate receptor in odorant recognition (Identified from AC brain transcriptome data) [48]	Midgut
14.	Glycine R - Glycine Receptor	Multiple tissues	Multiple tissues	Inhibit neurotransmission (Identified from AC brain transcriptome data) [49]	Midgut
15.	Akt Kinase - Protein kinase B	Fat body, ovary	Ovary	Activation of TOR pathway [11]	Ovary
16.	CYP31A41-20E hydroxylase (20E synthesizing enzyme)	Ovary	Fat body and ovary	Ovary and oocyte development [50]	Ovary
17.	STPK – Serine threonine protein kinase	Multiple	Multiple	Multiple physiological processes [51]	Ovary
18.	PI4-Kinase	Multiple	Multiple	Multiple physiological processes (Identified from	Ovary

				AC brain transcriptome data)	
19.	Calcitonin Receptor	Malphigian tubule	Malphigian tubule	Regulation of diuresis [45][52]	Malphigian tubule
20.	KDNaCa Exchanger	Malphigian tubule	Malphigian tubule	Regulate fluid secretion and diuresis [45]	Malphigian tubule
21.	V-Type ATPase	Malphigian tubule	Malphigian tubule	Regulate membrane potential and diuresis [45]	Malphigian tubule

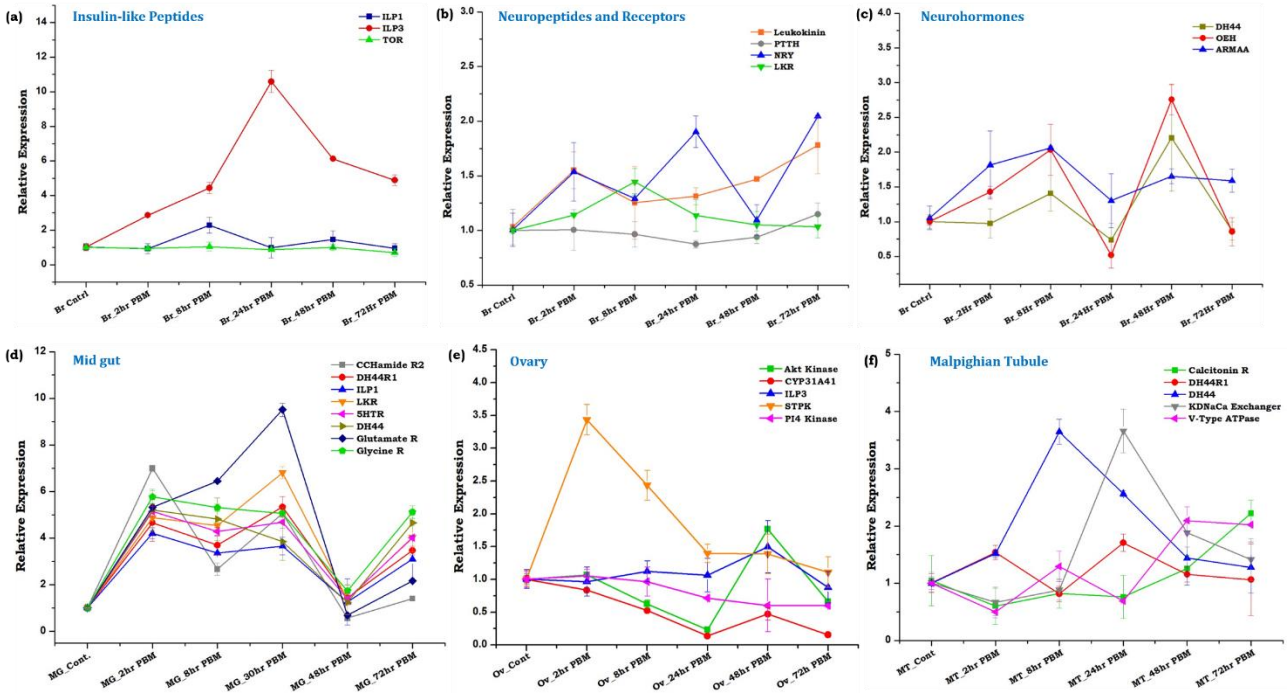


Fig. 4: <u>Innate physiological status differentially modulates tissue specific neuro-modulator transcripts expression</u>: (a - c) Transcriptional expression profiling of Insulin-like-peptides, neuropeptides, neurohormones and its receptor genes in the brain tissue during metabolic switch. The brain tissue was collected from 5-6-day old naïve sugar fed mosquitoes (Br_cont) followed by blood feeding and collection of the same at 2hr post blood meal (2hr-PBM), 8hr-PBM, 24hr-PBM, 48hr-PBM, 72hr-PBM; **(d)** Relative expression profiling of a subset of neuromodulator genes in the midgut of naïve and blood fed mosquitoes at the same time point described above; **(e)** Transcriptional profiling of genes involved in signal transduction during vitellogenesis in the ovary; **(f)** Relative gene expression analysis of diuresis related genes in the malphigian tubule of naïve and blood fed mosquitoes.

Gut, the '*second brain*' communicate the nutritional status through neurotransmitter synthesis: It is well evident from studies in vertebrates and fruit fly Drosophila, that communication between gut and brain has paramount effect in shaping optimal health [21] [53][19], but, no evidence exists on gut-brain axis communication in mosquitoes. A noteworthy observation of a blood meal induced modulation of the neuromodulator expression in the mosquito gut for longer duration invigorate us to presume the existence of bi-directional gut-brain axis communication (Fig. 5a). To further establish a proof-of-concept, we followed LC/MS based

absolute quantification of different neurotransmitters (NT) in the brain as well as in gut of naïve and blood fed mosquitoes (Fig. 5a).

The obtained data revealed that in naïve sugar fed mosquitoes, though brain serves as the primary source of NT synthesis, midgut also have substantial amount of NT (Fig. 5b). However, blood feeding caused a drastic shift of the NT level in the midgut than in the brain (Fig. c, d). Notably, we observed an unpredictable increment of most NT except glutamic acid, tyrosine and tyramine in the gut. Whereas, the brain tissue showed notable decrease in majority of the NT synthesis except histamine, tyrosine and tryptophan (Fig. 5c). We also observed that tyrosine amino acid exclusively induced in the brain after blood feeding, but remains below threshold level in the gut (Fig. 5c, d). Although the effect of tyrosine enrichment in the brain is intriguing, however, undetectable level of tyrosine in the gut supports previous finding that the scavenging of toxic tyrosine from the gut is essential for the safeguard journey of blood fed mosquitoes [54]. A substantial body of literature suggests that the biogenic amines such as dopamine and serotonin are the critical regulator of feeding, host seeking and cognitive functions [3,55–59]. Thus, an increase in the precursor molecules of dopamine i.e. tyrosine in the blood fed mosquito's brain may improve the cognitive power of mosquitoes, evidencing the existence of memory signature for host-seeking and blood feeding behavioral activities (Fig. 5c). Similarly, enrichment of tryptophan, a precursor of serotonin may favor to minimize the host-seeking behavioral activities of gravid females (Fig. 5c) [60]. Additionally, ~25-fold upregulation of GABAergic neurotransmission upon blood feeding in the midgut highlights its possible functionality in the regulation innate immune response by activating the autophagy response as a result of gut flora expansion following blood feeding (Fig. 5d) [61].

Although, the emerging knowledge in vertebrates and insect fly indicate that gut also serves as a major source of multiple neurotransmitters in parallel to the brain [62,63], but the mechanism of nutrition dependent NT modulation remains unclear [64]. Especially, in mosquitoes our understanding of complex nature of blood meal digestion and gut-brain axis communication is obscure. Thus, an unusual observation of thousand-fold increase in the level of histidine, serine, aspartic acid and tryptophan in the blood fed mosquito's gut emanated few key questions: 1) whether increased level of amino acid in the gut during blood meal digestion may acts as a NT? 2) do blood meal induced proliferation of the gut microbiota has any effect on NT dynamics? and 3) do the gut endo-symbionts of mosquitoes has any impact on gut-brain axis communication? (Fig. 5e).

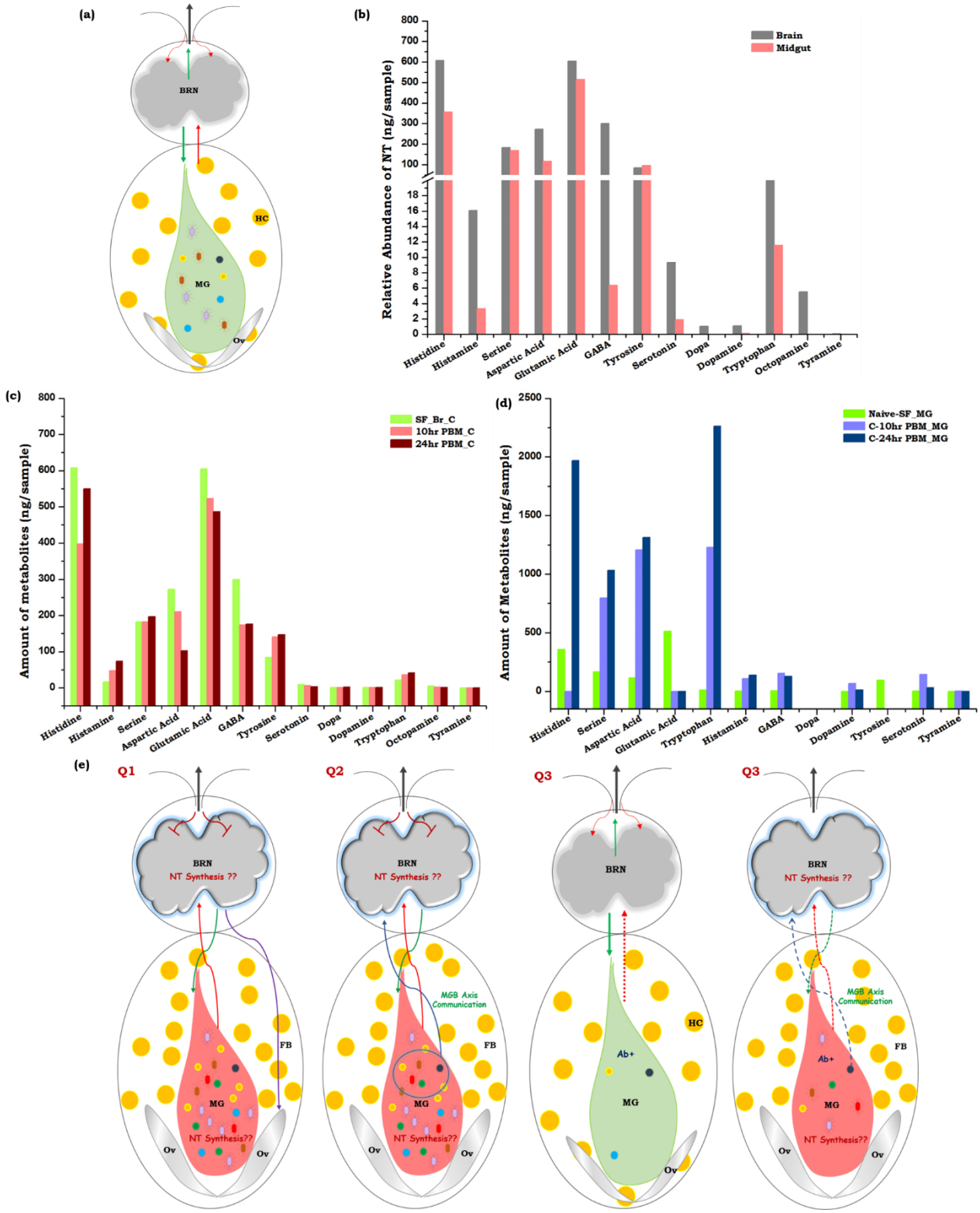


Fig. 5: Gut-Brain-Axis (GBA) communication and neurotransmitter (NT) estimation in the mosquito *An. Culicifacies*: (a) Hypothesis of GBA communication in mosquitoes; **(b)** Comparative analysis of NT abundance in the brain and midgut; **(c)** Absolute quantification of NT level in the brain of naïve and blood fed mosquitoes; **(d)** Blood meal induced change in the NT abundance in the gut tissue; **(e)** Pictorial presentation of the existence GBA communication after metabolic switch in mosquitoes. Q1: After blood feeding external/olfactory response of brain ceased. Upon stimulation through vagus pathway (red arrow) brain may engage to manage multiple functions occurring in distant tissues, such as midgut (green arrow), ovary (purple arrow). Thus, we questioned whether increased level of amino acid in the gut during blood

meal digestion may acts as a NT. Q2: Did blood meal induced increased gut flora (different colored shapes indicate diverse microbial flora) has any role in GBA communication in mosquitoes. Q3: Did the gut microbial population in mosquitoes has any role in microbiome-gut-brain axis (MGB) communication. BRN: Brain; MG: midgut; FB: Fat body; Ov: Ovary; Ab+: Antibiotic positive/treated.

Symbiotic gut flora influence alters gut-brain axis communication: The mechanism of gut-brain axis communication not only involve neuronal stimulation through vegus nerve but also include gut microbiota mediated regulation of gut endocrine system and biochemical pathways [20,65,66]. Previous literature suggests that mosquitoes has wide diversity of gut endo-symbionts affecting mosquito immunity, blood meal digestion and ecological adaptation [67,68]. It has also been reported that ingestion of protein rich blood meal favours rapid enrichment of gut microbiota [23] but how it affects the nexus of communication between gut and brain remains elusive. Thus, for conceptual understanding of microbiota-gut-brain-axis network influencing mosquito's behavioral physiology, we performed an absolute quantification of the potent neuroactive molecules i.e. neurotransmitters and compared their level in the gut and brain of naïve and antibiotic treated mosquitoes.

Aseptic non-blood fed mosquito showed a significant elevation of tryptophan and consequent downregulation of serotonin in both gut and brain (Fig. 6a, b). Corroborate with previous finding, we correlate that depletion of microbial flora may significantly alter tryptophan metabolism and delimit the de-novo-synthesis of serotonin, resulting in increased tryptophan concentration in the gut and brain [69]. Additional observation of a notable increase in histidine and histamine level in both gut and brain established that gut bacteria removal may influence systemic level of amino acid concentration (Fig. 6a, b).

In order to test how blood feeding to aseptic mosquitoes' influence gut-brain axis communication, again we quantified and compared neuro-transmitters level from antibiotic treated blood fed mosquitoes. Similar pattern of NTs synthesis was observed but the level of modulation is heightened in antibiotic treated blood fed gut and brain compared to naïve blood fed mosquitoes (Table 2, Fig. S2). To further support the above observation, we monitored and compared the expression pattern of neurotransmitter receptor genes (Glycine R, glutamate R, serotonin R, dopamine R), insulin-like-peptide and one of the junction protein gene (lachesin) in the gut and brain of naïve vs antibiotic treated mosquitoes (Fig. 6c). Consistent with NT quantitative data, the respective receptor genes also showed significant difference in their abundance throughout the gut-brain axis. We also noticed differential expression pattern of ILP3, ARMAA (Aromatic-L-amino-acid decarboxylase/serotonin synthesizing enzyme) and lachesin transcript between naïve and antibiotic treated mosquitoes undergoing metabolic switch event (Fig. 6c).

Together these findings provide a novel evidence that gut commensals are key modulators for the synthesis of major neuroactive molecules, and thus establish an active microbiome-gut-brain axis communication. Though, it is yet to clarify that how this cross-talk directly influences brain specific mosquitoes' mood and cognition. But, our data strongly suggest that a bi-directional gut-brain axis communication is key to manage complex gut immune-physiological responses and blood meal digestion process in blood fed insects.

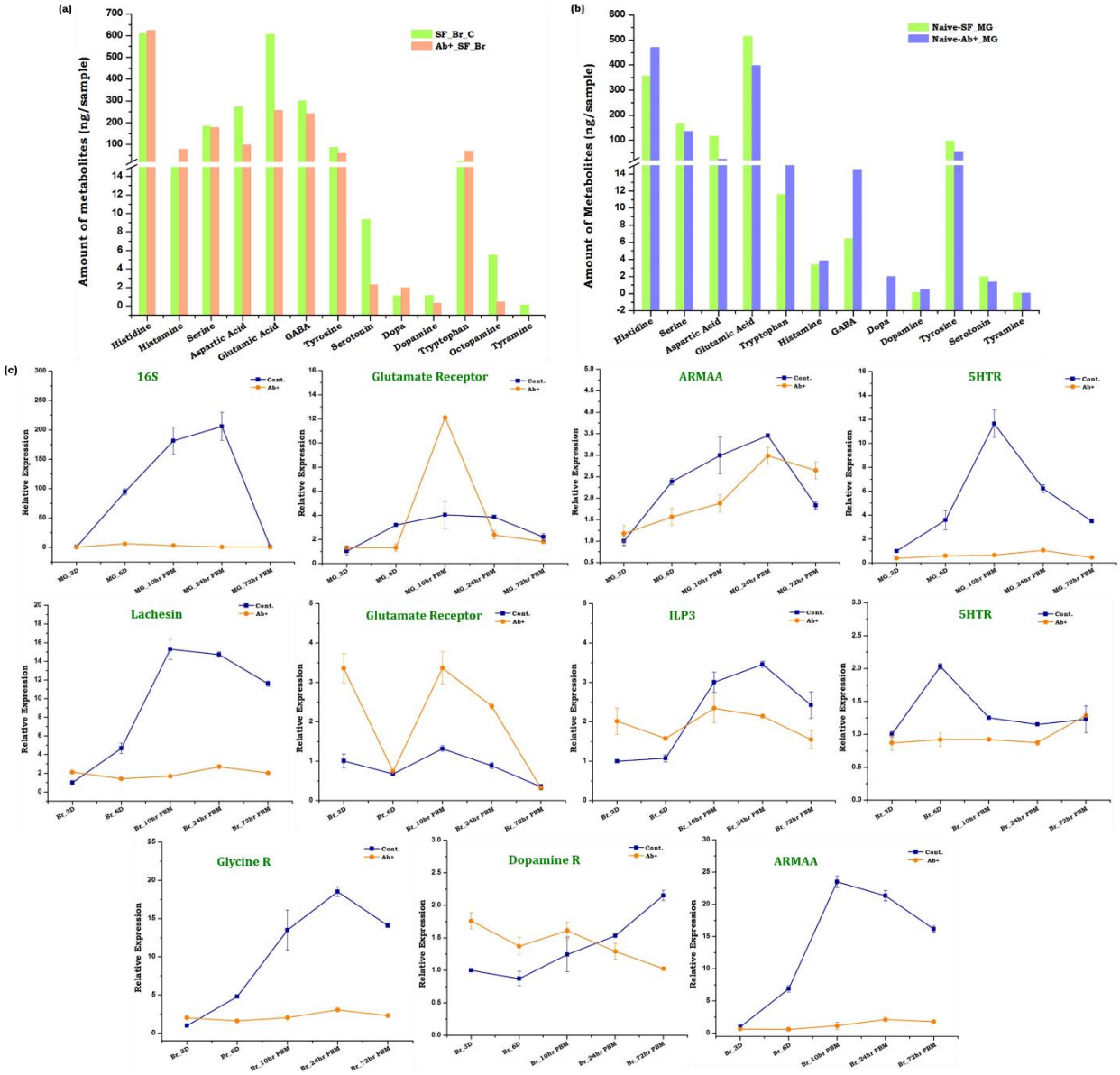


Fig. 6: <u>Establishing Microbiome-Gut-Brain-Axis (MGB) communication in mosquitoes:</u> (a) Absolute quantification of the neurotransmitters (NT) in the brain tissues of naïve sugar fed and antibiotic treated mosquitoes; **(b)** Quantitative estimation of the neurotransmitters (NT) in the gut tissues of naïve sugar fed and antibiotic treated mosquitoes; **(c)** Relative expression profiling of 16S gene to show population of microbial flora and other neuro-transcripts in the gut and brain of naïve and antibiotic treated mosquitoes undergoes metabolic switch.

Table 2: Quantitative estimation of 13 different neurotransmitters in the brain and 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 highly suppressed Internal Standard signal

Basal immunity may favour optimal brain function:

The immune system plays a crucial role in maintaining brain health by protecting it from both external and internal stress. Since, central nervous system and the immune system are the most energy consuming organs, we consider that the immune system may play important role to overcome the blood meal induced metabolic stress such as oxidative stress, osmotic stress, elevated levels of dietary heme molecules. To trace the possible linkage of brain-immune function we identified and catalogued a total of 913 immune transcripts from brain tissue transcriptome data (Fig. 7a). Among the 18 classified immune family proteins, autophagy, CLIP-domain serine proteases and peroxidases were observed the most predominant accounting more than 50% of the total immune transcripts. Furthermore, a comparative transcript abundance analysis showed that blood meal may cause a moderate change in the immune transcript expression (Fig. 7b). Slight up-regulation of peroxidases and CLIP-domain serine protease in blood-fed mosquito brain suggested that these immune transcripts may prevent brain tissue from oxidative stress-induced damage and facilitate its recovery (Fig. 7b). Further, functional analysis of the immune transcripts in the central nervous system may unravel the novel regulatory mechanism of the immune genes to maintain the brain in shape.

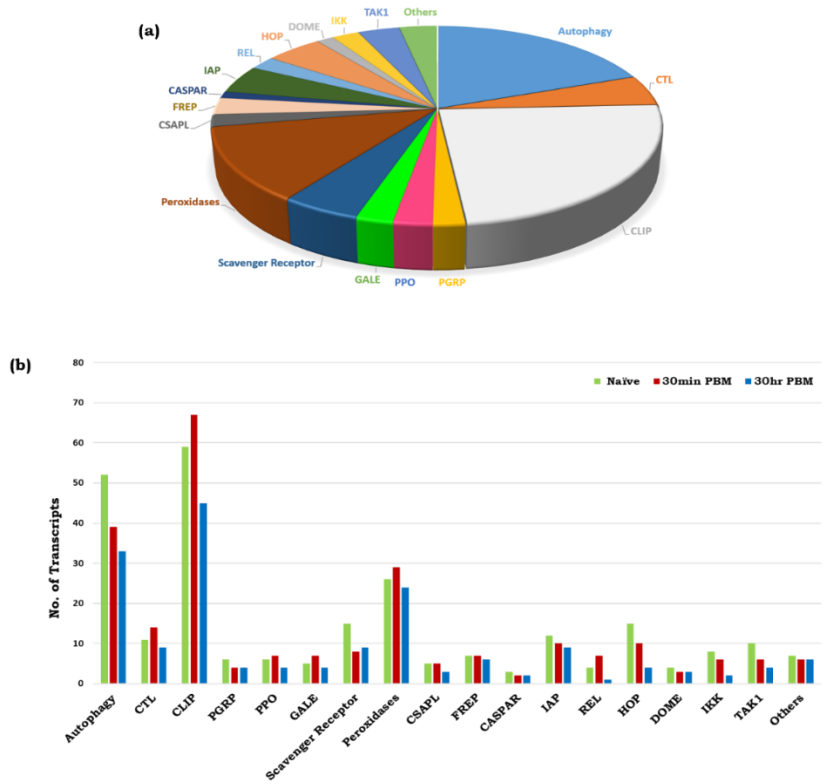


Fig. 7: <u>Molecular cataloguing of brain-specific immune transcripts</u>: (a) Molecular catalogue of the different class of immunity genes expressed in the brain tissue; **(b)** Differential expression pattern of the brain immunome as determined by the number of sequences appeared in each RNA-Seq data of naïve and blood-fed mosquito brain.

Discussion: Hematophagous insects, are evolved with complex nature of blood feeding behaviour. Resolving the molecular architecture of tiny brain and understanding the complexity of neuro actions during blood feeding by adult female mosquitoes remains challenging [3]. Using comprehensive RNA-Seq analysis, here we decoded the administrative role of the central nervous system and established a functional correlation of gut-brain axis in the mosquito *An. culicifacies*.

An exclusive induction of oxidation-reduction family proteins and a comparative pathway analysis predicts that blood meal enhances mitochondrial function and magnifies brain's energy metabolic activities. Evidences from *Drosophila* and vertebrates also suggest that an altered metabolic physiology influences a cross-talk between brain and peripheral tissues for the maintenance of systemic energy homeostasis [15,53,70–73]. To perform this action, a continuous neuronal stimulation is required, which consequently increases the energy demand of the brain [72,74]. An enrichment of fructose-mannose metabolic pathway provide an evidence of escalated energy metabolism in blood fed mosquitoes brain [75]. Next, a persistent elevation of PGC-1 and parallel enrichment of oxoglutarate dehydrogenase gene indicates the active mitochondrial function in the blood fed brain. Furthermore, the unique appearance of pentose phosphate pathway and glutathione peroxidase transcript (Oxidation-reduction category gene), along with the upregulation of CLIP-domain serine proteases and peroxidases immune transcripts may attribute to scavenging of ROS generated due to enhanced mitochondrial activity. Blood meal induced expression of amino acid transporters and trehalose transporter indicated that both trehalose and

amino acids may serve as a raw material for enhanced energy metabolism. Together, these data indicated that blood meal induced hyper energy metabolism may favor brain engagement and active functioning, which involves restoration of neuronal membrane potential, neurotransmitter recycling and axo-dendritic transport, to supervise other tissues to manage physiological homeostasis [70,76,77].

A spatial and temporal expression data of neuro-transmitter receptors and intracellular signalling molecule provided an indirect evidence that internal nutritional stimulus may accelerates brain functioning, possibly to manage distant organs task. To further support our hypothesis, we profiled a selected class of neuromodulators, neuropeptides and neuro-hormone genes expression in the brain and correlate their impact on distant organs. Corresponding to the innate physiological status, we observed a time dependent expression change of the respective transcripts in the brain, and other targeted tissues of mosquito such as midgut, Malpighian tubule and ovaries. But, in turn how this transcripts expression pattern reinstate brains action still remains unknown. Recent studies in *Drosophila* suggests leukokinin neuropeptide regulate protein diet induced post-prandial sleep and minimized movement [78]. We observed a transient increase in leukokinin and its receptor gene in the brain, and a sustained upregulation of leukokinin receptor gene in the gut till 30hrs of blood feeding. These data support the idea that until the blood meal get digested in the gut, brain may undergo 'food coma' and restricts external communication, but actively engaged to manage inter-organ communications. Compared to brain, a significant modulation of neuro-modulators in the gut, further suggested a specialized ability of gut to serve as "second brain" possibly to share and minimize the function of the primary brain [19]. Taken together, these data provide the first molecular evidence of a bidirectional 'gut-brain-axis' cross-talk during metabolic switch in mosquitoes.

The above observations prompted us to further clarify the mode of communication between gut and brain in mosquitoes. Neurotransmitters including both biogenic amines and amino acids are well known endogenous chemicals, which influence rapid inter-organ signal transmission and decision making abilities [62,65]. Therefore, to support and strengthen our hypothesis of metabolic switch induced gut-brain axis communication, we quantified the level of neurotransmitters secreted from both gut and brain tissues. When compared to the naïve sugar fed status, an unusual and paramount shift from brain to gut was observed for almost all the neurotransmitters level after blood feeding. A significant upregulation of aspartic acid, glutamic acid, histidine and histamine level in blood fed mosquitoes gut and brain may be a consequence of rapid degradation of protein rich blood meal in the mosquito gut [79]. We correlate that blood meal induced activation of vitellogenesis process may sequester majority of amino acids to nurture the eggs [50]. Remaining fraction of amino acids either serve as energy reservoir in the fat body [50] or functions as neurotransmitters, possibly to regulate gut-brain-axis communication, though further studies needed to prove the presumptions.

Appreciably, a recent term 'psycobiotics', which is focused to decode the influential effect of microbiome-gut-brain axis communication, is common to vertebrate neurobiology, but insects' community are least attended [66,80]. A limited research on vertebrates and fruit fly indicated that gut microbiota influences multiple behavioural physiologies such as host metabolism, appetite, mood, sensory perception and cognition [81–85]. However, the studies on mosquitoes' gut-

symbionts is predominantly limited to their impact on parasite growth and their potentiality for para-transgenic approaches [86]. Though, yet it is unclear whether rapid proliferation of gut flora also influence gut-neuro actions in mosquitoes. But a noteworthy modulation of gut neurotransmitters reinforces us to propose that gut microbiota may play pivotal role in gut-brain axis communication and endocrine regulation, a possible mechanism to overcome the metabolic switch induced stress response.

To test the above hypothesis, we disrupted the gut symbionts by providing antibiotic diet supplement to the newly emerged mosquitoes for 4-5 days. Surprisingly, aseptic adult female mosquitoes showed an assertive feeding behaviour towards a vertebrate host. Though, defining the molecular basis of this unusual behaviour is still a quest of understanding. But, our primary quantification data showed a significant difference in the gut and brain neurotransmitter abundance between the naïve sugar fed and germ free mosquito groups. It is therefore attractive to speculate that gut bacteria can sense the nutritional demand of mosquitoes and gut flora removal may enhance the cravings for protein rich diet. Recent studies in insect flies also demonstrated that gut commensal bacteria and the composition of dietary amino acid supplement greatly influence in shaping behavioural responses such as food choice decision and olfactory guided foraging decision [84,87,88]. Furthermore, higher abundance of histamine in the brain and GABA in the gut of antibiotic treated mosquitoes may be accountable to elicit/provoke the arousal in adult female mosquitoes. This may be a possible reason of enhanced aggressiveness towards host-seeking/blood feeding behavior either directly through neuro-stimulation or indirectly through vagal pathway [89,90]. Blood meal uptake causes a rapid gut-microbial proliferation, which consumes crucial amino acids to synthesize the building blocks of bacterial cell wall components [91,92]. Following blood meal to aseptic mosquitoes, a multi-fold upregulation of serine and glutamic acid provide an evidence of limited usage of the respective amino acids due to restricted growth of microbial flora (Table 2) (Fig. S2).

Taken collectively the current investigation provide a novel insight that how mosquitoes tiny brain is evolved to switch its function from external communication (pre-blood meal host-seeking and host selection) to internal multiple organ communication (post-blood meal physiological homeostasis) for the fitness of the mosquitoes. Additionally, it is the first evidence which emphasize the crucial role of gut endosymbionts in microbiome-gut-brain axis communication. Our conceptual framework may be valuable to modify mosquitoes' olfactory perception and cognition through the alteration of gut-bacteria.

Funding statement: Laboratory work was supported by Indian Council of Medical Research (ICMR), Government of India (No.3/1/3/ICRMR-VFS/HRD/2/2016) and Tata Education and Development Trust (Health-NIMR-2017-01-03/AP/db). Tanwee Das De is the recipient of ICMR-Post Doctoral Fellowship Scheme (3/1/3/PDF(18)/2018-HRD). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data Deposition: The sequencing data were deposited to National Center for Biotechnology Information (NCBI) Sequence Reads Archive (SRA) system (BioProject accessions: PRJNA555826; BioSample accessions: SRR9853884 for Ac-Br-SF, SRR9853885 for Ac-Br-30Min, and SRR9853883 for Ac-Br-30hrs).

Authors contribution statement: TDD, KCP, YH, RKD: Conceived and designed the experiments: TDD, ST, DS, VS, PS, CR, SK, ST, JR; RD; contributed to design and performing the experiments, data acquisition, writing and editing; TDD, YH, KCP, RKD: data analysis and interpretation, data presentation, contributed reagents/ materials/Analysis tools, wrote, reviewed, edited, and finalized MS. All authors read and approved the final manuscript.

Competing interest statement: The authors declare no conflict of interest

Acknowledgement: We thank insectary staff members for mosquito rearing. We also thank Kunwarjeet Singh for technical assistance in laboratory. Finally, we are thankful to Xceleris Genomics, Ahmedabad, India for generating NGS sequencing data. We thank to CCAMP Metabolomics Facility for performing neurotransmitter (NT) analysis. We are grateful to Padma Ramakrishnan, Technology Scientist, Mass Spectrometry Facility - Centre for Cellular and Molecular Platforms, Bengaluru for her inputs in the manuscript by providing the details of LC/MS based NT analysis and also thank her for troubleshooting during our study.

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