



HAL
open science

Circadian and age-related variations of amino acids levels in *Drosophila* brains: correlations and descriptive dimensions

Sandrine Parrot, Jacob Crehan, Chloé Aman, Philippe de Deurwaerdère, Matthew Thimgan, Laurent Seugnet

► To cite this version:

Sandrine Parrot, Jacob Crehan, Chloé Aman, Philippe de Deurwaerdère, Matthew Thimgan, et al.. Circadian and age-related variations of amino acids levels in *Drosophila* brains: correlations and descriptive dimensions. 2025. hal-04950597

HAL Id: hal-04950597

<https://hal.science/hal-04950597v1>

Preprint submitted on 17 Feb 2025

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL
open science

Circadian and age-related variations of amino acids levels in *Drosophila* brains: correlations and descriptive dimensions

Sandrine Parrot, Jacob Crehan, Chloé Aman, Philippe de Deurwaerdère, Matthew Thimgan, Laurent Seugnet

► To cite this version:

Sandrine Parrot, Jacob Crehan, Chloé Aman, Philippe de Deurwaerdère, Matthew Thimgan, et al.. Circadian and age-related variations of amino acids levels in *Drosophila* brains: correlations and descriptive dimensions. 2025. hal-04950597

HAL Id: hal-04950597

<https://hal.science/hal-04950597v1>

Preprint submitted on 17 Feb 2025

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Circadian and age-related variations of amino acids levels in *Drosophila* brains: correlations and descriptive dimensions

Sandrine Parrot^{1*}, Jacob Crehan², Chloé Aman³, Philippe De Deurwaerdère³, Matthew Thimgan⁴, Laurent Seugnet^{2*}

¹ Université Claude Bernard Lyon 1, INSERM, Centre de Recherche en Neurosciences de Lyon CRNL U1028 UMR5292, NeuroDialyTics Unit, F-69500, Bron, France

² Université Claude Bernard Lyon 1, INSERM, Centre de Recherche en Neurosciences de Lyon CRNL U1028 UMR5292, Team WAKING, F-69500, Bron, France

³ University of Bordeaux, CNRS, Institut des Neurosciences Intégratives et Cognitives d'Aquitaine INCIA CNRS UMR5287, F-33000, Bordeaux, France.

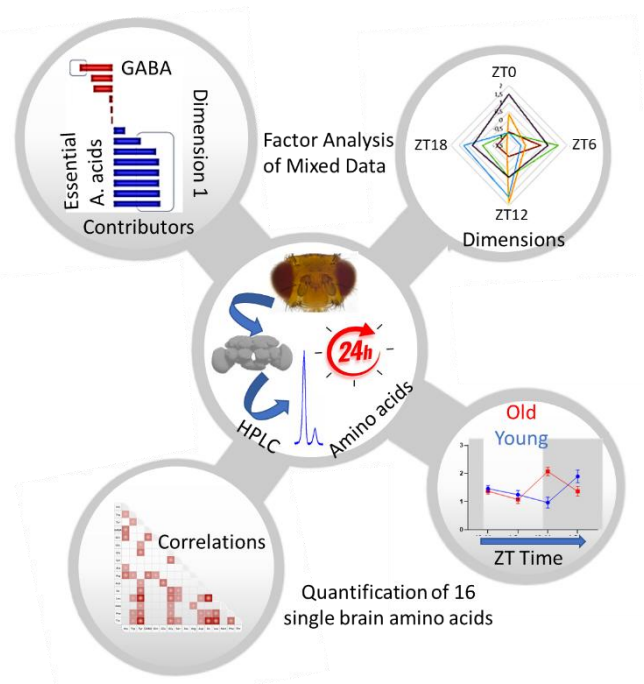
⁴ Missouri Science and Technology, MO 65401, Rolla, MO, USA

Corresponding authors

*Mailing address: Dr Laurent Seugnet and Dr Sandrine Parrot, Université Claude Bernard Lyon 1, INSERM U1028 CNRS, UMR5292, Centre de Recherche en Neurosciences de Lyon (CRNL), 95 Bld Pinel, 69500 Bron, France. E-mails: laurent.seugnet@inserm.fr; sandrine.parrot@univ-lyon1.fr

Abstract

A comprehensive view of whole-brain amino acid levels hold the potential to provide valuable insights into the brain's state, given the mutual interconnections through metabolism, food intake, and neurotransmission. We tested this concept by evaluating free amino acid levels in single *Drosophila* brains across 24h and at two different ages. A large proportion of these amino acids displayed time-of-day variations, and a subset exhibited age-dependent variations. Cross-correlation analysis of the datasets confirmed broad time-of-day and age dependent interconnections between amino acids. Factor Analysis of Mixed Data revealed further data structuration along key amino acids. For example, 50% of the variance could be accounted by an inverse coupling between gamma-aminobutyric acid and several essential amino acids during the active phase, linking food intake and sleep. This proof of concept emphasizes the value of combining multivariate analysis to whole-brain amino acid levels evaluation, shedding potentially new light on sleep-wake regulation and aging.



Introduction

Metabolism in the central nervous system and in the brain in particular, differs in a number of ways from that of peripheral organs. It exhibits high-intensity, notably driven by the need to sustain the energy demands essential for maintaining ionic balance, enabling neuronal firing, supporting the lipid and protein composition of its extensive membranous structures, and providing precursors for neurotransmitter synthesis. In this context, among the numerous metabolites involved in brain metabolism, proteinogenic amino acids are particularly noteworthy for a number of reasons: 1) they are of course pivotal for protein synthesis but also involved in energy metabolism, nitrogen homeostasis, and lipid biosynthesis, 2) they cannot accumulate in intracellular storage systems, such as glycogen and lipid droplets; 3) a subset of them, the 9 essential amino acids, can only be supplied by diet or protein degradation, such that the uptake and transport of these amino acids are crucial 4) they are necessary for the synthesis of direct precursors of neurotransmitters (like dopamine and GABA), or 5) are themselves major brain neurotransmitters (like glutamate and glycine).

Amino acid synthesis, transport, use and degradation are strongly interconnected, especially in the brain. For example, branched chain amino acids, through transamination, play a crucial role in the glutamate/GABA-glutamine cycle and can influence the excitatory/inhibitory balance. Glutamate and aspartate are the substrates of the mitochondrial antiporter system that imports glutamate from the cytosol into the matrix and exports aspartate from the matrix to the cytosol, enabling the regeneration of NADH, promoting in particular high levels of ATP synthesis and lipid biosynthesis. The precursors of monoamine neurotransmitters: phenylalanine, histidine, tryptophan and tyrosine compete for the same transporter, the Large neutral Amino acid Transporter 1 (LAT-1), to enter the cells, and in particular to penetrate the blood-brain barrier, where they also compete with branched-chain amino acids. The import of those essential neutral amino acids requires the export of other amino acids, notably glutamine, since LAT-1 is an antiporter system.

Given these interconnections, evaluating the complete panorama of amino acid levels in the brain holds the potential to provide valuable insights into the brain's state. Indeed, in the last few decades, classical as well as transcriptomic, proteomic and metabolomic studies have indicated that, among others, brain amino acids levels and metabolism are highly dynamic, being modulated by the circadian clock, sleep-wake states, food intake, and across lifespan, by aging [1–5]. In such studies, however, amino acids are examined as integrated components of general metabolism, alongside a wide range of other metabolites, and not as a distinct metabolic network. Most amino acids are controlled by homeostatic processes, even as they participate in multiple metabolic pathways. Regardless of potential quantitative changes in their concentrations in the brain, their involvement in specific pathways may shift across time and conditions. This would imply that the relationships between amino acids can be addressed via multivariate analyses to report interconnectedness and extract meaningful insights from such data.

To address this model, our study adopts a novel approach, treating amino acids as elements of an interconnected network that reflects, at least partially, the broader metabolic system. If successful, this type of analysis could leverage relatively simple datasets to uncover previously unrecognized processes influencing brain physiology. We tested this hypothesis by evaluating free amino acid levels in single *Drosophila* brains across 24h and at two different chronological ages in *Drosophila*. Since food intake and wakefulness are higher during the light phase, this allowed us to assess their combined influence [6,7], as well as aging in young and old adult flies.

Results and discussion

Wild-type male flies, aged 6 days (young) and 41 days (old), were individually dissected to collect their brains every 6 hours under baseline conditions. Collection occurred at four time points: the start (Zeitgeber or ZT 0) and midpoint (ZT 6) of the light phase, and the start (ZT 12) and midpoint (ZT 18) of the dark phase. Male flies exhibit peak activity at the start and end of the light phase, with food intake predominantly occurring during the first half of the day [7,8]. The dark phase serves as the primary sleep period for *Drosophila*, during which they spend most of their time sleeping [6,9,10]. Whole brain free amino acids levels were evaluated in each brain using high performance liquid chromatography (HPLC) analysis. We detected 16 different amino acids, including GABA. Valine could not be detected in all samples, proline was not quantifiable and cysteine and lysine were not studied. Consistent with previous findings, the quantified amino acids showed a broad range of average abundances, with alanine (Ala) being significantly more prevalent than the others (Figure 1A). This is likely due to Ala's role as a key energy metabolite in the brain [11,12]. In contrast, the essential amino acids methionine (Met), tryptophan (Trp), and phenylalanine (Phe) were the least abundant. Excluding Ala, amino acid concentrations ranged within one order of magnitude, between 0.1 and 1 ng per brain, making them relatively abundant and easily detectable metabolites in single brain homogenates. We observed fluctuations in the levels of Glutamine (Gln), Ala, Threonine (Thr), Tyrosine (Tyr), Met, Leucine (Leu), isoleucine (Ileu), GABA, Serine (Ser), and Aspartate (Asp) depending on the time of day. This indicates that a majority of brain amino acids (10 out of 16) are influenced by factors such as vigilance state, food intake, or circadian clock-dependent processes (Figure 1, B-E, Figures S1 and S2). Interestingly, the highest GABA levels were detected at the start of the dark phase, coinciding with the period of deepest sleep in flies (Figure 1D). This aligns with the well-established role of GABAergic inhibition in sleep regulation in *Drosophila*, particularly during the first half of the dark phase, similar to its function in mammals [13–15]. In contrast, the essential or semi-essential amino acids Leu, Met, and Tyr were more abundant during the light phase, indicating a possible link to food intake and/or wakefulness (Figure 1C, 1E). Specifically, Tyr serves as a precursor for dopamine, while Leu can significantly impact the GABA/Glutamate (Glu) ratio [1,16]. We also observed an age-related effect on the abundance of Gln, Glycine (Gly), Tyr, and Ala. This effect was particularly pronounced for Gln: like GABA, Gln levels were higher at the start of the night in young flies, though with a much greater amplitude of change (approximately a 100% increase, Figure 1 D). However, this variation was entirely absent in older flies, suggesting that it is age-dependent. In line with this observation, Gln and GABA levels are strongly correlated in young flies but not in older flies (Figure 2). Age-related changes in glutamine levels linked to circadian time and aging have been noted in a previous metabolomic study [5]. These changes may stem from age-related alterations in sleep (e.g [17]) and, subsequently, in the GABA-Glu-Gln cycle.

To further examine the interconnection between the different amino acids across time and aging, we conducted a systematic cross-correlation analysis of the data set. As shown in Figure 2, pooling the data from young and old flies revealed numerous cross correlations. Some were seen across ages such as GABA-Arginine (Arg), Ser-Gly or the correlation between the essential amino acids Phe, Leu, Ileu, Tyr, Thr, Trp. Several others were age-dependent such as Gln-GABA previously mentioned and only significant in young flies or Ala-GABA only significant in old flies. Interestingly, the number of cross-correlations also tended to fluctuate with time of day, being rarest at the beginning of the light phase, reaching a maximum at the beginning of the dark phase and decreasing thereafter, both in old and young flies (Figure 2). Some of the correlations could originate from close metabolic links, such as the direct interconvertibility between Ser and Gly, the Gln-Glu-GABA cycle or from co-transport: Ser-Gly or Phe-Leu-Ileu-Trp. The decreased number of correlations at ZT 0

may be attributed to an increased supply of essential amino acids in the fly brain due to feeding, which temporarily disrupts steady-state processes.

Finally, we conducted a Factor Analysis of Mixed Data (FAMD) across the whole dataset to identify key patterns or relationships among the variables. This analysis revealed that almost 80% of variance could be reduced in three latent dimensions (Table 1). This type of multivariate analysis not only reveals the contributions of quantitative variables, as in a traditional principal component analysis, but also incorporates qualitative variables (see Supplemental Figure S3 for the first two main dimensions and table 2). In order to avoid multiple biplot graphs (10 in total to compare Dim 1 vs 3, Dim 1 vs 4, ..., Dim 4 vs 5, etc.), we generated an alternative representation based on a mixture of treemap and population graph (Figure 3A). The largest coordinates in absolute values could orient the potential interpretations of the dimension (Supplemental Figure S3, [18]). The major dimension, accounting for nearly 50% of the variance, was strongly influenced by essential amino acids, which were symmetrically opposed to GABA - another significant contributor. This dimension was greatest at ZT 6, which, along with ZT 0, is a time point closely linked to the day's largest food intake and wakefulness (Figure 3B, red line) [6,7] as discussed previously (Figure 1D-E). This suggests that the availability of essential amino acids, particularly branched-chain amino acids, and certain monoamine precursors (Histidine, Tyr, Trp), may be functionally coupled with GABA, in accordance with previous evidence [1,19]. The dimension characteristics are only modestly altered with age as shown in Figure 3B (red line), suggesting that they reflect age-independent processes. In the second dimension, GABA again emerged prominently, but this time in conjunction with Arg (Figure 3A). The strong contribution from ZT12 (Figure 3B, yellow line, see also Table 2) in this dimension is coherent with the highest levels of GABA and Arg observed at these time points (Figure 1D and S2). This finding aligns with the established association between sleep and GABA neurotransmission [20] as well as GABA levels [3]. Additionally, Arg has also been shown to increase during the dark phase in *Drosophila* whole body metabolomics [3], an observation that might tentatively relate to sleep-dependent nitrogen metabolism [21]. Interestingly, this dimension, similar to the first, seemed relatively unaffected by age (Table 2) and may primarily represent vigilance-state-dependent processes that remain unchanged with age. In contrast, the lesser 3 dimensions were differently represented in young and old flies. The third dimension (13%) was predominantly influenced by Ser and Ala (Figure 3A), two glucogenic amino acids involved in cellular exchanges and serving as energy metabolites. Serine is also a precursor of acetylcholine, a neurotransmitter heavily used in the *Drosophila* brain. The qualitative variables reveal a clear difference in time contribution between young and old flies (see ZT 6 vs ZT 12 in Figure 3B) possibly reflecting age-related changes in neurotransmission and energy metabolism across circadian time. The fourth dimension (12%) grouped the following major quantitative contributors: Glu, its precursor Gln, and Asp, possibly linked to ATP and lipid biosynthesis or Glu signalling (Figure 3A). ZT 12 and ZT 18 emerged as prominent qualitative contributors (Table 2, Figure 3B) that were differentially distributed between old and young flies. Gln may play a key role in this difference, as it lacked the pronounced increase in levels observed in young flies at the start of the night (ZT 12, Figure 1D). The fifth dimension was more challenging to interpret biochemically due to the diverse contributions of smaller quantitative variables (Figure 3A). Interestingly this dimension was dramatically influenced by age (Table 2) regardless of ZT (Figure 3B). This surprising observation regarding 5th dimension would deserve to be further investigated in light of other datasets. Altogether, the dimensions provided by multivariate analysis extend the interpretation of the data beyond single amino acids variations and confirm the potential of this combined strategy.

In summary, these findings provide a proof of concept demonstrating the potential of assessing free amino acid levels in the brain. Their abundance makes them relatively easy to detect and identify using HPLC analysis, enabling a detailed view of brain states across the time of day and lifespan. Cross-correlational analysis and a variant of principal component analysis further highlighted the striking interconnectedness of amino acid availability, offering interpretative insights that warrant further investigations, provided that a sufficient number of individual flies could be sampled. These results lay the groundwork for more in-depth studies combining sleep data with amino acid measurements from the brain and haemolymph in individual flies, aiming to unravel the metabolic regulation underlying sleep and circadian rhythms across lifespan.

Material and Methods

Fly stocks and husbandry

Male flies from the Canton *S Drosophila melanogaster* wild type stock were bred and maintained as adults on standard food containing yeast, molasses, sucrose and corn meal, in 12h:12h light:dark schedule and 60% humidity. Before being dissected, flies were kept in groups of 10 in individual vials. Flies were transferred to new vials every week. ZT 0: correspond to the beginning of the light phase at 10-11 am.

Tissue extraction in single brain samples

Each brain was quickly dissected from 48 cold-anesthetized flies and frozen in individual PCR tube in 1 μ L of ringer solution at -70°C until being processed for tissue content analysis. More precisely, 3 μ L of ice-cold 0.1 mol/L perchloric acid containing 1.34 mmol/L EDTA and 0.05%, w/v sodium bisulfite were added to each sample and samples were then sonicated in an ultra-sonic bath for 12 min. The homogenates still in PCR tubes were centrifuged at 15,000 g for 20 min at $+4^{\circ}\text{C}$ in 1.5-mL tubes filled with water to prevent the PCR tubes from fragmentation during the centrifugation process. The supernatants were analyzed for amino acid content on the same day.

Chemicals for amino acid analysis

Naphthalene-2,3-dicarboxaldehyde (NDA) and sodium cyanide (NaCN) were purchased from Fluka (Buchs, Switzerland) and acetonitrile from Merck (Darmstadt, Germany). DL-alanine (Ala), DL-arginine (Arg), DL-asparagine (Asn), DL-aspartate (Asp), DL-glutamine (Gln), glycine (Gly), DL-histidine (His), DL-isoleucine (Ile), DL-leucine (Leu), DL-methionine (Met), DL-phenylalanine (Phe), DL-serine (Ser), DL-threonine (Thr), DL-tryptophan (Trp), DL-tyrosine (Tyr), DL-valine (Val), DL-glutamate (Glu), and gamma-aminobutyric acid (GABA), trifluoroacetic acid (TFA), boric acid, and sodium tetraborate were purchased from Sigma-Aldrich (St. Louis, MO, USA), methanol was obtained from VWR. Ultrapure water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). Each standard solution of 1 mmol/L amino acid was stored at 4°C as aliquots in 0.1 mol/L hydrochloric acid (diluted from a 37% solution, Merck) or water.

Analysis of single brain amino acid content using HPLC-LIFD

Tissue amino acid levels were quantified using high performance liquid chromatography coupled with laser induced fluorescence detector (HPLC-LIFD) using chromatographic conditions adapted to microvolumes from [22] in a low-pressure binary gradient mode. The HPLC system consisted of a Prominence degasser, a high-pressure LC-30 AD pump (pump A), a low-pressure LC-20 pump (pump B), a 20- μ L mixer, and an SIL-30AC autosampler

from Shimadzu. It was coupled to a ZETALIF laser induced fluorescence detector (Picometrics, France). The mixing of the mobile phases from pumps A and B and fluorescence detection were performed at room temperature before injection and after separation, respectively, whereas the separations were carried out at 30°C in an oven. The column used has the following features: ChromaNik, SunShell C18 90Å 3.5µm, 2.1 x 100mm (ref. CN-CB9961). Excitation was performed using a 410-nm diode laser (Melles Griot, USA) and the emission wavelength was set at 490 nm. The detection was carried out at the outlet of the column in a fused-silica capillary serving as detection cell (i.d. 75 µm, OD 375 µm, stripped out of its polyimide layer at 50 cm (effective length), total length 60 cm, connected to a PE tubing towards waste).

The day of the analysis, the samples were thawed, diluted 10-fold in water (1/9, v/v) and derivatized to tag the amine moiety of the amino acids, as previously described [23]. The derivatization reagents were added off-line in 10-µL diluted samples, by adding 4 µL of a solution made with 120 µL of borate/NaCN solution (mixing solution [110:22, v/v] of 500 mmol/L borate buffer pH 8.7, and 87 mmol/L NaCN in water), 60 µL of ultrapure water, and, in the end, 60 µL of NDA solution (5.85 mmol/L in acetonitrile/water, 50:50, v/v). The reaction took place at room temperature directly in the PCR collection tubes for 10 minutes. The PCR tubes were then placed capless in the autosampler and maintained at 4°C until injection

An injection volume of 2 µL was used, and separations were conducted at a flow rate of 0.203 mL/min. Gradient elution was performed using acidified water with 0.1% TFA as mobile phase A and methanol as mobile phase B. The gradient conditions were as follows: from 0.00 to 2.89 minutes, 50% for phase B; from 2.89 to 13.5 minutes, a linear increase from 50% to 90% of phase B; a linear decrease from 90% to 50% of phase B from 13.5 to 14.5 minutes, and thereafter maintained at 50% until the end of the run. The acquisition time was 20.5 minutes, with a system pressure of approximately 290 bars. Chromatograms were acquired at a rate of 10 Hz using Lab Solutions software. Retention times for His, Arg, Asn, Gln, Ser, Asp, Gly, Glu, Thr, Tyr, Ala, GABA, Trp, Met, Val, Phe, Leu, and Ile were determined as 3.4 min, 4.9 min, 5.3 min, 5.8 min, 6.5 min, 6.9 min, 7.4 min, 7.7 min, 8.1 min, 8.5 min, 8.8 min, 9.3 min, 10.5 min, 10.7 min, 11.3 min, 11.4 min, 12.1 min, 12.3 min, respectively. Analyte concentrations were determined by comparing chromatographic peak areas with calibration curves derived from a mixture of 18 synthetic standards.

One sample was lost during the derivatization step. For the remaining 47 samples, the Asn peak in drosophila brain samples was not pure so that the quantification was not possible, while Gly, Phe and Val levels were below the limit of detection in one, 3, and 33 samples, respectively. Final concentrations of each amino acid were expressed as ng of amino acid per single brain sample.

ANOVA and Kruskal-Wallis analyses

The normality of the data for each amino acid was checked by a Shapiro-Wilks test. Normal datasets were analyzed using a 2-way ANOVA. The remaining datasets were analyzed using Kruskal-Wallis tests (all ages). The significance was set at $p < 0.05$. GraphPad Prism and the StatEL plugin in excel were used as statistical software. For these analyses, amino acid concentrations were regarded as outliers when their values were outside the interval of 'mean±2SD'. Four concentrations with values just below the 'mean-2SD' limit were kept to avoid losing analysis power.

Analysis of correlations

Correlations, performed using Spearman's correlation test, were made on the content of each amino acid. Correlations were then considered as significant at $p < 0.05$.

Factor analysis of mixed data

Factor analysis of mixed data (FAMD), a variant of PCA considering both quantitative and qualitative data, provides less new dimensions due to the integration of residual variances brought by the qualitative variables while preserving the characteristics/nature/properties of the first dimensions [24]. Fourteen quantitative variables (amino acids measured, except Val, Phe and Gly due to the existence of missing values for these three variables) were analyzed using the snowCluster package in the 'click button' R-based Jamovi 2.3.21 software. Five dimensions cumulating 100% of the variance, and obtained with no rotation factor, were generated. The data are gathered in Supporting Information (Excel file).

Author Contributions

S.P. worked on the study design, data collection, data analysis and writing the manuscript. J.C. worked on data collection and data analysis C. A. and P.D.D worked on data analysis, M. T. and L.S. worked on the study design, data collection, and writing the manuscript.

Funding Sources

The present study was supported by the Université Claude Bernard Lyon 1, the Institut de la Santé et de la Recherche Médicale (Inserm), the Missouri University of Science and Technology and the Université of Bordeaux,

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Supporting Info Circadian monitoring of AA - Parrot et al (PDF)

Database-Crehan-et-al-2023-fly-article-Supporting-Information (XLS).

Table 1. Eigenvalue decomposition. Eigenvalue and (cumulative) proportion of explained variance per new dimension following a factor analysis of mixed data on 14 amino acids (GABA, Ala, Asp, Met, His, Thr, Ile, Leu, Glu, Gln, Ser, Tyr, Trp, Arg) and two qualitative variables (young vs old; 4 times of the day)

Dimension	Eigenvalue	% of Variance	Cumulative %
1	6.98	49.65	49.7
2	2.33	16.57	66.2
3	1.81	12.87	79.1
4	1.64	11.64	90.7
5	1.30	9.26	100.0

Table 2. Coordinates of the two qualitative variables (age:young vs old; Time: ZT 0, ZT 6, ZT, 12, ZT 18) across the dimensions using a factor analysis of mixed data on 14 amino acids (GABA, Ala, Asp, Met, His, Thr, Ile, Leu, Glu, Gln, Ser, Tyr, Trp, Arg) in single brain samples. Values close to or above 0.5 (highlighted) represent the variable of high contribution in the considered dimension.

Across dimensions - coordinates	Dim 1	Dim 2	Dim 3	Dim 4	Dim 5
Age	0.03487	0.00327	0.04445	0.05801	0.57508
Time	0.15744	0.47853	0.25910	0.51380	0.11545

Figure Legends

Figure 1. A. Average relative abundance of 16 tissue amino acids from single brain samples (n=47, mean +/- SD, outliers excluded). B. Tissue contents of amino acids involved in general metabolism (B), precursors of neurotransmitters (C), neurotransmitters and related metabolite (D), and essential (E), across the day from four subgroups of 24 young or 23 old flies. The other amino acids are shown in Figures S1 & S2 in the Supporting Information. (ZT 0 = 10-11 am, ZT 6 = 4-5 pm, ZT 12 = 10-11 pm and ZT 18 = 4-5 am,)

Figure 2. Analysis of correlations with significant Pearson's values between the 16 amino acids in the two subgroups (n=24 young vs n=23 old flies) (left) or depending on the period of the day (ZT 0 = 10-11 am, ZT 6 = 4-5 pm, ZT 12 = 10-11 pm and ZT 18 = 4-5 am,) (right). H: His; W: Trp; Y: Tyr; γ : GABA; Q: Gln; E Glu; G: Gly; S: Ser; A: Ala; R: Arg; D: Asp; I: Ile; L: Leu; M: Met; F: Phe; T: Thr.

Figure 3. Factor Analysis of Mixed Data (FAMD) on the neurochemical dataset of 14 quantitative variables (amino acids, except Val, Gly and Phe due to the existence of at least one missing values for these three compounds) from 47 individuals. A. Treemap of the five dimensions generated by FAMD, including the contributions of each quantitative variables in each new dimension. Variables with factor contribution >0.5 are emphasized in order to help interpret the dimensions generated by the multivariate analysis. B. Radar chart representing the average of the individuals' coordinates across the five dimensions during the time of the day (ZT 0 = 10-11 am, ZT 6 = 4-5 pm, ZT 12 = 10-11 pm and ZT 18 = 4-5 am).

References

- [1] Li H, Seugnet L. Decoding the nexus: branched-chain amino acids and their connection with sleep, circadian rhythms, and cardiometabolic health. *Neural Regen Res* 2025;20:1350–63. <https://doi.org/10.4103/NRR.NRR-D-23-02020>.
- [2] Bourdon AK, Spano GM, Marshall W, Bellesi M, Tononi G, Serra PA, et al. Metabolomic analysis of mouse prefrontal cortex reveals upregulated analytes during wakefulness compared to sleep. *Sci Rep* 2018;8. <https://doi.org/10.1038/s41598-018-29511-6>.
- [3] Malik DM, Rhoades SD, Kain P, Sengupta A, Sehgal A, Weljie AM. Altered Metabolism during the Dark Period in *Drosophila* Short Sleep Mutants. *J Proteome Res* 2024;23:3823–36. <https://doi.org/10.1021/acs.jproteome.4c00106>.
- [4] Malik DM, Paschos GK, Sehgal A, Weljie AM. Circadian and Sleep Metabolomics Across Species. *Journal of Molecular Biology* 2020;432:3578–610. <https://doi.org/10.1016/j.jmb.2020.04.027>.
- [5] Schwarz JE, Sengupta A, Guevara C, Barber AF, Hsu CT, Zhang SL, et al. Age-regulated cycling metabolites are relevant for behavior. *Aging Cell* 2024;23:e14082. <https://doi.org/10.1111/ace1.14082>.
- [6] Andretic R, Shaw PJ. Essentials of Sleep Recordings in *Drosophila*: Moving Beyond Sleep Time. In: Young MW, editor. *Methods in Enzymology*, vol. 393, Academic Press; 2005, p. 759–72. [https://doi.org/10.1016/S0076-6879\(05\)93040-1](https://doi.org/10.1016/S0076-6879(05)93040-1).
- [7] Murphy KR, Deshpande SA, Yurgel ME, Quinn JP, Weissbach JL, Keene AC, et al. Postprandial sleep mechanics in *Drosophila*. *eLife* n.d.;5. <https://doi.org/10.7554/eLife.19334>.
- [8] Xu K, Zheng X, Sehgal A. Regulation of feeding and metabolism by neuronal and peripheral clocks in *Drosophila*. *Cell Metab* 2008;8:289–300. <https://doi.org/10.1016/j.cmet.2008.09.006>.
- [9] Shaw PJ, Cirelli C, Greenspan RJ, Tononi G. Correlates of sleep and waking in *Drosophila melanogaster*. *Science* 2000;287:1834–7. <https://doi.org/10.1126/science.287.5459.1834>.
- [10] Hendricks JC, Finn SM, Panckeri KA, Chavkin J, Williams JA, Sehgal A, et al. Rest in *Drosophila* is a sleep-like state. *Neuron* 2000;25:129–38. [https://doi.org/10.1016/s0896-6273\(00\)80877-6](https://doi.org/10.1016/s0896-6273(00)80877-6).
- [11] Rabah Y, Francés R, Minatchy J, Guédon L, Desnous C, Plaçais P-Y, et al. Glycolysis-derived alanine from glia fuels neuronal mitochondria for memory in *Drosophila*. *Nat Metab* 2023;5:2002–19. <https://doi.org/10.1038/s42255-023-00910-y>.
- [12] Volkenhoff A, Weiler A, Letzel M, Stehling M, Klämbt C, Schirmeier S. Glial Glycolysis Is Essential for Neuronal Survival in *Drosophila*. *Cell Metabolism* 2015;22:437–47. <https://doi.org/10.1016/j.cmet.2015.07.006>.
- [13] Dissel S, Angadi V, Kirszenblat L, Suzuki Y, Donlea J, Klose M, et al. Sleep restores behavioral plasticity to *Drosophila* mutants. *Curr Biol* 2015;25:1270–81. <https://doi.org/10.1016/j.cub.2015.03.027>.
- [14] Liu S, Lamaze A, Liu Q, Tabuchi M, Yang Y, Fowler M, et al. WIDE AWAKE Mediates the Circadian Timing of Sleep Onset. *Neuron* 2014;82:151–66. <https://doi.org/10.1016/j.neuron.2014.01.040>.
- [15] Chung BY, Kilman VL, Keath JR, Pitman JL, Allada R. The GABAA Receptor RDL Acts in Peptidergic PDF Neurons to Promote Sleep in *Drosophila*. *Curr Biol* 2009;19:386–90. <https://doi.org/10.1016/j.cub.2009.01.040>.
- [16] Yudkoff M. Interactions in the Metabolism of Glutamate and the Branched-Chain Amino Acids and Ketoacids in the CNS. *Neurochem Res* 2017;42:10–8. <https://doi.org/10.1007/s11064-016-2057-z>.
- [17] Koh K, Evans JM, Hendricks JC, Sehgal A. A *Drosophila* model for age-associated changes in sleep:wake cycles. *Proc Natl Acad Sci U S A* 2006;103:13843–7. <https://doi.org/10.1073/pnas.0605903103>.
- [18] Parrot S. Do neurochemicals reflect psychophysiological dimensions in behaviors? A transdisciplinary perspective based on analogy with Maslow’s needs pyramid. *ACS Chemical Neuroscience*, in Revision n.d.

- [19] Li H, Aboudhief S, Parrot S, Scote-Blachon C, Benetollo C, Lin J-S, et al. Pallidin function in *Drosophila* surface glia regulates sleep and is dependent on amino acid availability. *Cell Rep* 2023;42:113025. <https://doi.org/10.1016/j.celrep.2023.113025>.
- [20] Chaturvedi R, Emery P. Fly into tranquility: GABA's role in *Drosophila* sleep. *Curr Opin Insect Sci* 2024;64:101219. <https://doi.org/10.1016/j.cois.2024.101219>.
- [21] Bedont JL, Kolesnik A, Pivarshev P, Malik D, Hsu CT, Weljie A, et al. Chronic sleep loss sensitizes *Drosophila melanogaster* to nitrogen stress. *Current Biology* 2023;33:1613-1623.e5. <https://doi.org/10.1016/j.cub.2023.03.008>.
- [22] Siri N, Lacroix M, Garrigues J-C, Poinsot V, Couderc F. HPLC-fluorescence detection and MEKC-LIF detection for the study of amino acids and catecholamines labelled with naphthalene-2,3-dicarboxyaldehyde. *Electrophoresis* 2006;27:4446–55. <https://doi.org/10.1002/elps.200600165>.
- [23] Sauvinet V, Parrot S, Benturquia N, Bravo-Moratón E, Renaud B, Denoroy L. In vivo simultaneous monitoring of γ -aminobutyric acid, glutamate, and L-aspartate using brain microdialysis and capillary electrophoresis with laser-induced fluorescence detection: Analytical developments and in vitro/in vivo validations. *ELECTROPHORESIS* 2003;24:3187–96. <https://doi.org/10.1002/elps.200305565>.
- [24] Pagès J. Analyse factorielle de données mixtes. *Revue de Statistique Appliquée* 2004;52:93–111.