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Loss of temporal coherence in the circadian metabolome across multiple tissues during ageing in mice

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Abstract

Circadian clock function declines with ageing, which can aggravate ageing-related diseases such as type 2 diabetes and neurodegenerative disorders. Understanding age-related changes in the circadian system at a systemic level can contribute to the development of strategies to promote healthy ageing. The goal of this study was to investigate the impact of ageing on 24-h rhythms in amine metabolites across four tissues in young (2 months of age) and old (22–25 months of age) mice using a targeted metabolomics approach. Liver, plasma, the suprachiasmatic nucleus (SCN; the location of the central circadian clock in the hypothalamus) and the paraventricular nucleus (PVN; a downstream target of the SCN) were collected from young and old mice every 4 h during a 24-h period ($n = 6–7$ mice per group). Differential rhythmicity analysis revealed that ageing impacts 24-h rhythms in the amine metabolome in a tissue-specific manner. Most profound changes were observed in the liver, in which rhythmicity was lost in 60% of the metabolites in aged mice. Furthermore, we found strong correlations in metabolite levels between the liver and plasma and between the SCN and the PVN in young mice. These correlations were almost completely abolished in old mice. These results indicate that ageing is accompanied by a severe loss of the circadian coordination between tissues and by disturbed rhythmicity of metabolic processes. The tissue-specific impact of ageing may help to differentiate mechanisms of ageing-related

Abbreviations: BIC, Bayesian information criterion; EDTA, ethylenediaminetetraacetic acid; FDR, false discovery rate; GABA, gamma-aminobutyric acid; LOD, limit of detection; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; ZT, zeitgeber time (ZT0 = lights on).

disorders in the brain versus peripheral tissues and thereby contribute to the development of potential therapies for these disorders.

KEYWORDS
ageing, amine metabolism, chronobiology, circadian rhythms, hypothalamus, liver, metabolomics, plasma

1 | INTRODUCTION

Life expectancy at older age has increased substantially in the past three decades (Mathers et al., 2015). Ageing often comes with a debilitating decline of bodily functions, including marked changes in the daily rhythms that characterise our behaviour and physiology (Hood & Amir, 2017). These ageing-related changes include fragmented sleep–wake cycles, shifted and blunted hormonal rhythms and dampened metabolic rhythms (Hood & Amir, 2017; Panagiotou et al., 2017; Sato et al., 2017).

These 24-h rhythms in bodily functions are coordinated by the circadian clock, the endogenous timing system consisting of a central clock in the suprachiasmatic nucleus (SCN) of the hypothalamus and peripheral clocks in nearly all cells of the body. The SCN receives and processes light information to adjust the phase of the circadian system and synchronises peripheral clocks to the external light/dark cycle. One main route by which the SCN relays the timing signal to other brain areas and peripheral organs is via the paraventricular nucleus (PVN) in the hypothalamus. This pathway controls circadian rhythmicity in several neuroendocrine axes, such as corticosteroid hormones, as well as in autonomic outputs (Lu & Kim, 2022). Importantly, the SCN also receives feedback from the rest of the body, for example, about physical activity, metabolic state and stress.

Ageing is known to compromise the functioning of the circadian system, although not all elements in the system are equally affected (Buijink & Michel, 2021). For example, in the SCN, the molecular clock seems largely unaffected in ageing (Buijink et al., 2020; Kolker et al., 2003; Yamazaki et al., 2002), while cellular properties, such as calcium signalling, ion channels and action potential frequency, are severely affected (Farajnia et al., 2012; Nakamura et al., 2011). In peripheral tissues, circadian rhythms in genome-wide gene expression are dampened and acrophases (i.e. peak timing) are altered in ageing, suggesting weakened circadian output (Wolff et al., 2023). Disrupted circadian rhythms are thought to contribute to ageing-associated disease states, such as inflammation, atherosclerosis, obesity, type 2 diabetes and Parkinson's as well as Alzheimer's disease (Castanon-Cervantes et al., 2010; Finger & Kramer, 2021; Leng et al., 2019; Schilperoort et al., 2020). Therefore, a better understanding of the changes in circadian coordination in physiology that occur during ageing can contribute to the development of targeted interventions and diagnostic tools for age-related diseases.

Omics approaches offer a way to gain insight into the relationship between ageing and the circadian system on a systemic level. While transcriptomics can reveal ageing-related changes in clock-controlled gene expression (Chen et al., 2016), metabolomics can offer insight into the impact of ageing on 24-h rhythms at a biochemical level. Indeed, metabolomics studies have shown the influence of environment and behaviour on the function of the circadian system (Brown, 2016; Dallmann et al., 2012). For example, a high-fat diet has been shown to significantly affect 24-h rhythms in metabolite levels in the liver and to reduce the temporal relationship of metabolites between different tissues in mice (Dyar et al., 2018; Eckel-Mahan & Sassone-Corsi, 2013). Metabolomics have also been widely used to study the ageing process (Srivastava, 2019; Zierer et al., 2015).

A metabolite class of special interest in the context of ageing are amines, which include amino acids and their biogenic metabolites. Amines play fundamental roles in cellular metabolism and energy production, with some of them, such as dopamine, serotonin, and gamma-aminobutyric acid (GABA), serving as neurotransmitters (Plenis et al., 2019; Wu, 2009). Alterations in amine metabolism are associated with ageing-related diseases and longevity (Hofer et al., 2022; Santin et al., 2021; Toledo et al., 2017). Amines have also been implicated in the crosstalk between ageing and the circadian system (Acosta-Rodriguez et al., 2021). The biosynthesis of polyamines (i.e. putrescine, spermidine and spermine) was found to be under circadian control, and their decline in ageing was found to be related to a lengthening of circadian period (Zwighaft et al., 2015).

To obtain systems-level insight into the impact of ageing on the circadian coordination of amine metabolism across tissues, we used a targeted metabolomics approach to assess 24-h rhythmicity in abundance of amino acids and their biogenic metabolites in plasma and tissue of young and aged mice. In the brain, we sampled the SCN as the central circadian clock in mammals as well as the

PVN. Since SCN function is influenced by metabolic feedback and the PVN is essential for relaying SCN output, both areas are interesting targets for metabolomic studies. We also sampled tissue from the liver, which has one of the best studied peripheral circadian clocks and, together with data from plasma samples, promised to give insights in differential impact of ageing within the circadian system. Subsequently, we mapped how metabolite levels correlate within and among these tissues and how this is affected by ageing. Altogether, this provides a comprehensive overview of tissue-specific alterations in the temporal coherence of amine metabolism during ageing.

2 | MATERIALS AND METHODS

2.1 | Animals and housing

Male C57BL/6 mice were kept at the animal facility of the Leiden University Medical Center. The mice were held under a light/dark cycles with 12 h of light (150–200 lx; Osram Truelight TL, Osram GmbH, Munich, Germany) and 12 h of darkness in cages equipped with running wheels as a type of enrichment and with food and water provided ad libitum. Mice were initially housed in groups. Two to three weeks before tissue collection, they were housed individually until the day of tissue collection. The study was performed in accordance with the Dutch law on animal welfare, with a permit from the animal experiments committee Leiden (DEC 12250).

2.2 | Tissue collection

C57BL6 mice were sacrificed by decapitation at zeitgeber time (ZT) 2, 6, 10, 14, 18 and 22 (see Figure 1 for an overview of the experimental design; ZT 0 = lights on). Young mice were 2 months of age, and aged mice were between 22 and 25 months at the time of sacrifice. The liver was dissected, placed in a tube, snap-frozen in liquid nitrogen, and stored at −80°C until further processing. Trunk blood was collected immediately after decapitation and transferred to a blood collection tube with EDTA. Plasma samples were subsequently stored at −80°C until further processing. SCN and PVN tissue was collected and stored at −80°C until metabolite extraction. Sampling procedures of SCN and PVN were optimised to ensure minimal contamination by neighbouring brain nuclei as described previously (Buijink et al., 2018). The same animals were used to collect adipose tissue for a circadian lipidomic analysis to address an independent hypothesis (Held et al., 2021).

2.3 | Metabolite extraction and profiling

Metabolite profiling was performed at the biomedical metabolomics facility of the Leiden University (Leiden, The Netherlands). All samples were analysed on a platform covering 74 amino acids and biogenic amines. SCN and PVN samples were processed for metabolite extraction as described previously (Buijink et al., 2018). Liver tissue was freeze-dried and pulverised. Around 3 mg of dried liver tissue was weighed and placed in 2 ml tubes.

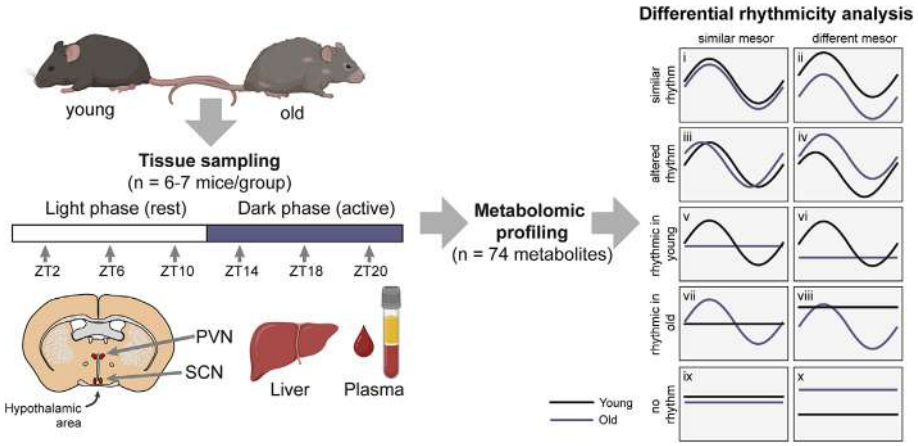

FIGURE 1 Experimental design. Tissue sampling from young and old mice was performed at six time points during the light/dark cycle. SCN, PVN, liver and plasma tissues were collected and processed for metabolomic profiling using LC-MS. After data pre-processing, differential rhythmicity analysis was performed using a model-selection approach to compare metabolite rhythmicity and levels in young and old mice. This results in 10 possible model categories, which are schematically represented; 'altered rhythm' (category iii and iv) refers to metabolite pairs with one of the cosinor parameters or both different between young and old mice.

Then, 500 µl of methanol–water (50:50 v/v) with a mix of 27 isotopically labelled internal standards was added to the sample. Liquid–liquid extraction was performed by adding 500 µl of chloroform to the samples. A Bullet Blender 24 (Next Advance, NY, USA) was used for further tissue homogenisation (2 × 2 min at intensity 7 with two 3.2 mm steel beads). After centrifugation, 400 µl from the top layer of methanol–water was transferred to a 1.5 ml tube and dried in a vacuum concentrator (Labconco, MO, USA). Plasma was processed for metabolite extraction by adding 30 µl of H_2O with a mix of 27 isotopically labelled internal standards and 75 µl of methanol to 5 µl of plasma. The samples were vortexed and centrifuged for protein precipitation. 105 µl of the top layer was transferred to a clean tube taken to dryness in a speedvac.

Following extraction, metabolites in the tissue samples were analysed by liquid chromatography and mass spectrometry (LC-MS/MS). The dried extracts from SCN, PVN, liver and plasma samples were reconstituted in borate buffer (SCN and PVN: 10 µl, liver and plasma: 70 µl; pH 8.5), then vortexed for 10 s and derivatised with AccQ-Tag reagent (SCN and PVN: 2.5 µl, Liver and plasma: 20 µl; Waters, Etten-Leur, the Netherlands). After incubation at 55°C for 30 min, 20% formic acid (SCN and PVN: 5 µl, liver and plasma: 10 µl) was added, and the samples transferred to a sample vial. Vials were placed in the autosampler tray where they were kept at 4°C until injection.

Subsequently, 1 µl of the sample was injected onto an Agilent 1290 Infinity II LC System (Santa Clara, CA, USA), on an AccQ-Tag Ultra column (Waters) with a flow rate of 0.7 ml/min over an 11 min. Gradient. The system was coupled to a triple quadrupole mass spectrometer (AB SCIEX Qtrap 6500, Framingham, MA, USA). Analytes were detected in the positive ion mode and monitored in multiple reaction monitoring (MRM) using nominal mass resolution. Data were processed using MultiQuant software for quantitative analysis (AB SCIEX, version 3.0.2). Samples were measured in random order. The integration of assigned SRM peaks was performed using Quanlynx software (Waters). Peak area was normalised by a matching internal standard using $^{13}C^{15}N$-labelled analogues of the amino acid or the closest-eluting labelled amino acid.

2.4 | Data processing

Subsequent data processing and analysis was performed in R (v 4.2.2) (R Core Team, 2022). Peak areas that were below three times the peak area of the blank samples were considered to be below the limit of detection (LOD). A metabolite was excluded from further analysis if more than 20% of samples were below the LOD for a given metabolite within a tissue. Remaining peak areas were divided by the peak areas of the internal standard. The liver samples were normalised to their sample weight. The data were then $\log_2$-tranformed. Data points that were more than three times the standard deviation from the mean of that metabolite within a tissue were considered to be outliers and removed. If more than 20% of the results from one sample met this criterium, the whole sample was considered to be compromised and removed.

2.5 | Data analysis

To compare 24-h rhythms in metabolite profiles in young and aged mice, differential rhythmicity analysis was performed as described previously (Atger et al., 2015; Kervezee et al., 2018). In short, a model selection approach was applied on the data from each metabolite from each tissue in which the fit of different linearised cosinor models was compared using the Bayesian information criterion (BIC) to select the optimal model among the set of models. This set comprised 10 models (visualised in Figure 1): (model i, ii) a 24-h rhythm in young and aged mice with shared cosinor parameters (i.e. similar amplitude and phase), (iii, iv) a 24-h rhythm in young and aged mice with one of the cosinor parameters or both altered (i.e. different in either amplitude and/or phase), (v, vi) a 24-h rhythm in young mice but not in aged, (vii, viii) a 24-h rhythm in aged mice but not in young, (ix, x) no 24-h rhythm in aged and young mice, assuming a shared mesor between young and aged mice (i.e. similar overall levels; model i, iii, v, vii, ix) or a different mesor (i.e. higher or lower overall levels; model ii, iv, vi, viii, x). The fit of the selected model was compared to the null model (model ix) using a log-likelihood ratio test to assess significance. Resulting p values were corrected for multiple testing using the Benjamini–Hochberg method at the tissue level (false discovery rate; FDR < 0.05). The cosinor coefficients from the selected models were used to calculate the amplitude and acrophases (Cornelissen, 2014). The number of rhythmic metabolites between young and aged mice was compared using chi-squared tests to test equal proportions.

Correlations between metabolite levels within and between tissues were quantified with Pearson correlations coefficient and visualised as heatmaps and circular plots. Within tissues, we calculated the correlation between the levels of each pair of metabolites (e.g. between spermine and dopamine in the liver and all other combinations). Between tissues, we calculated the correlation between the levels of all metabolite pairs

between each pair of tissues (e.g. between spermine in the liver and dopamine in the SCN). Correlations within and between tissues were considered relevant when $|rho| \geq 0.7$.

The metabolic profiles of Dyar et al. (2018) and our own study were compared by using a two-way ANOVA on z-scored metabolite levels over time. To this end, raw data from that publication (released under a CC BY 4.0 licence) was downloaded (Dyar, 2018). Since the sampling times in both studies were different (ZT 0, 4, 8, 12, 16, 20, 24 for Dyar et al. (2018) and ZT 2, 6, 10, 14, 18, 22 in the present study), 1 hour was added to the ZT from Dyar et al. (2018) and 1 hour subtracted from the ZT of our own study. The last time point of Dyar et al. (2018), that is, ZT24, was left out of the comparison. A significant interaction ($p < 0.05$) indicates a different 24-h metabolite profile.

3 | RESULTS

Using a targeted metabolomics panel on samples from liver, plasma, SCN and PVN tissue collected every 4 h over a period of 24 h from 36 young mice and 40 aged mice ($n = 6$–7 per sampling time point; Figure 1), we were able to detect 73 metabolites in liver, 66 metabolites in SCN and 61 metabolites in plasma and PVN (Dataset S1). The number of samples available for analysis per group, tissue and time point is shown in Table S1. Comparison of data from the SCN, liver and plasma from the young mice in our study to metabolomic data from these tissues in the control group previously published by Dyar et al. (2018) revealed a large degree of similarity, with comparable peak and trough times of metabolites that show a 24-h rhythm (Figures S1–S3). Statistical comparison of the two studies by time of day revealed no significant differences between the profiles for any metabolite in any tissue (all FDR-adjusted p values >0.05; Figures S1–S3), supporting the reproducibility of our results.

3.1 | Ageing affects 24-h rhythms in metabolite levels in a tissue-specific manner

To explore the effect of ageing on the temporal coordination of the metabolome, we used differential rhythmicity analysis to assign metabolites into different categories for each tissue depending differences in rhythmicity or overall abundance (Figures 2 and 3). Across metabolites and tissues, all selected models provided a significantly better fit than the null model (model ix in Figure 1; all FDR-adjusted p values <0.05; see Dataset S1).

In the liver of young mice, 51% of all detected metabolites ($n = 37$ out of 73) showed a daily rhythm in their level (Figure 2a). This percentage was significantly reduced in ageing ($n = 15$ out of 73, $\gamma^2(1) = 13.2$; $p = 0.0002$, test of equal proportions), with 25 metabolites losing rhythmicity and three metabolites gaining rhythmicity in ageing and 12 metabolites being rhythmic in both young and aged mice (Figure 2a). Most metabolites in the liver peak at night in both young and aged mice, between ZT12 and ZT24 ($n = 29/37$ in young and $n = 11/15$ in aged; Figure 2b). However, in young mice, a peak is present at ZT16 that is absent in aged mice. In addition, the abundance of 48 metabolites was increased in the liver of aged mice compared to young mice, while five had a decreased abundance (Figure 2a). A list of metabolites per differential rhythmicity category for the liver is given in Dataset S1, and a representative example for each category is shown in Figure 2c.

In plasma, 36% of the measured metabolites ($n = 22$ out of 61) showed a 24-h rhythm in young mice and 34% ($n = 21$ out of 61 in aged mice); hence, the number of rhythmic metabolites did not differ between young and aged mice ($\gamma^2(1) = 1$; $p = 1$, test of equal proportions), with 20 metabolites being rhythmic in both young and aged mice and two metabolites losing rhythmicity and one metabolite gaining rhythmicity in ageing (Figure 2d). In both young and aged mice, the majority of metabolites peak between ZT17 and ZT24 ($n = 16$ out of 22 in young and $n = 19$ out of 21; Figure 2e). Furthermore, the abundance of 17 metabolites was increased in plasma of aged mice compared to young mice, while 14 had a decreased abundance (Figure 2a,d). Seventy-three per cent ($n = 53$ out of 73) of metabolites in the liver and 51% ($n = 31$) of metabolites in plasma show a different level in ageing. A list of metabolites per model category for plasma is given in Dataset S1, and a representative example for each category is shown in Figure 2f.

For a small number of metabolites in the SCN and PVN, a daily rhythm was detected in their levels (Figure 3a,d). In the SCN, no effect of ageing was found on the number of rhythmic metabolites ($\gamma^2(1) = 1.08$; $p = 0.299$, test of equal proportions): 9% of the measured metabolites (n = 6 out of 66) were rhythmic in young and 17% ($n = 11$ out of 66) in aged mice. Five metabolites were rhythmic in both young and aged mice and one metabolite lost rhythmicity, while six gained rhythmicity (Figure 3a). Peak time was similar in young and aged SCN, with three out of five metabolites peaking between ZT18 and ZT20 in young and 10 out of 11 in aged mice (Figure 3b). Furthermore, the abundance of 23 metabolites was increased in the SCN of aged mice compared to young mice, while 14 had a decreased abundance. A list

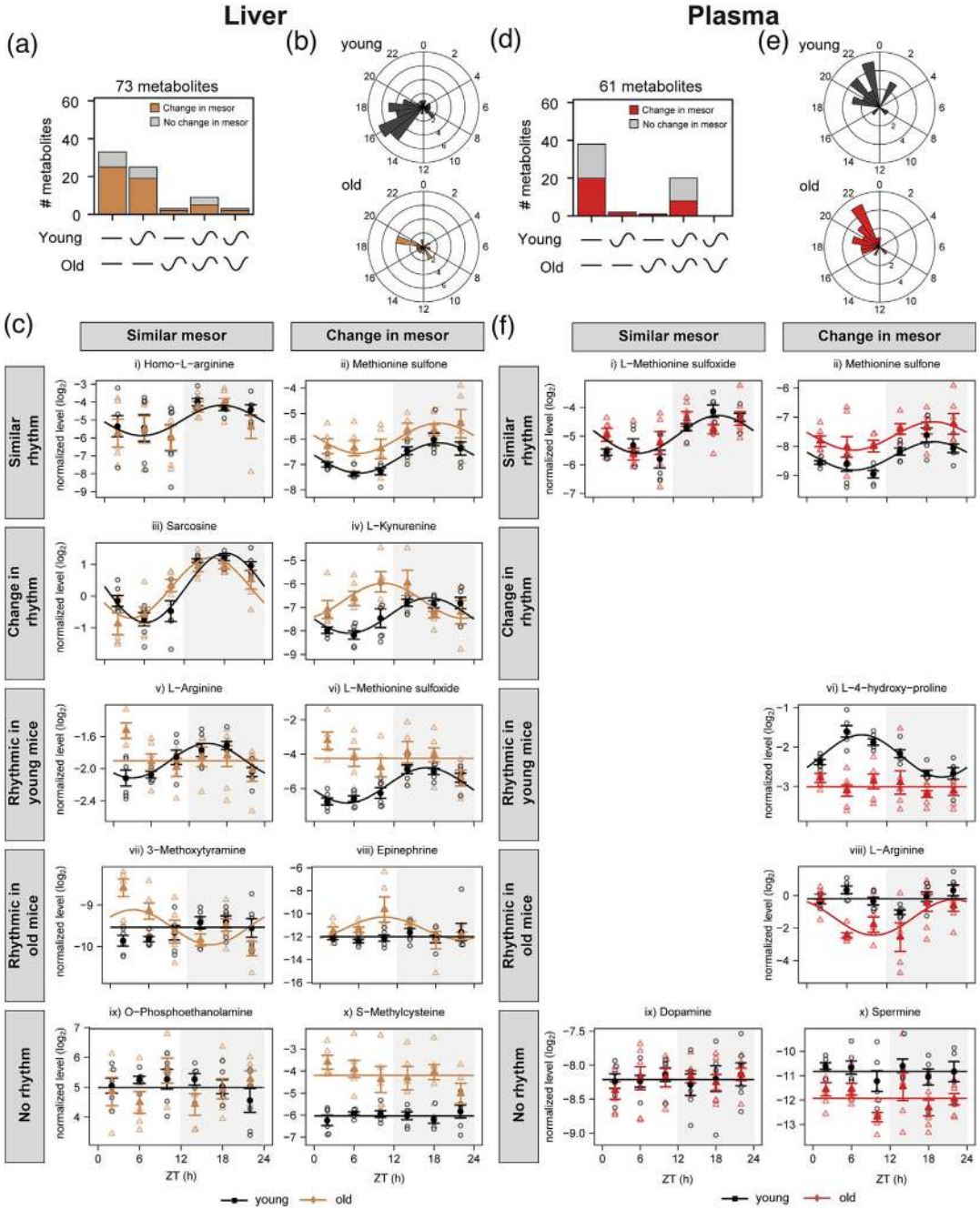

FIGURE 2 Metabolite rhythms and levels change in liver and plasma in the course of ageing. (a,d) Categorisation of metabolites into categories based on differential rhythmicity analysis in liver (a) and plasma (d). Bars show the number of metabolites per category: those that show a 24-h rhythm neither in the young nor in the old group; in the young group but not in the old group; in the old group but not in the young group; in both groups with similar amplitudes and peak times; or in both groups with different amplitudes and/or peak times. Differently coloured portions indicate the number of metabolites that have higher or lower levels in the old group compared to the young group. (b,e) Peak timing of rhythmic metabolites in the young (upper panel) and old (lower panel) group in liver (b) and plasma (c). (c,f) Representative examples of metabolites assigned to one of the 10 different model categories the selected models, based on changes in average levels and rhythmicity shown for liver (c) and plasma (f). Metabolite levels are represented in the $\log_2$ of the MS peak area divided by the internal standard. Data are shown as individual data points and as mean ± standard error of the mean. Lines depict model fits resulting from differential rhythmicity analysis. Grey areas mark the dark period (active phase), and light areas represent light period (resting phase). For plasma, no metabolites were assigned to model category iii, iv, v and vii.

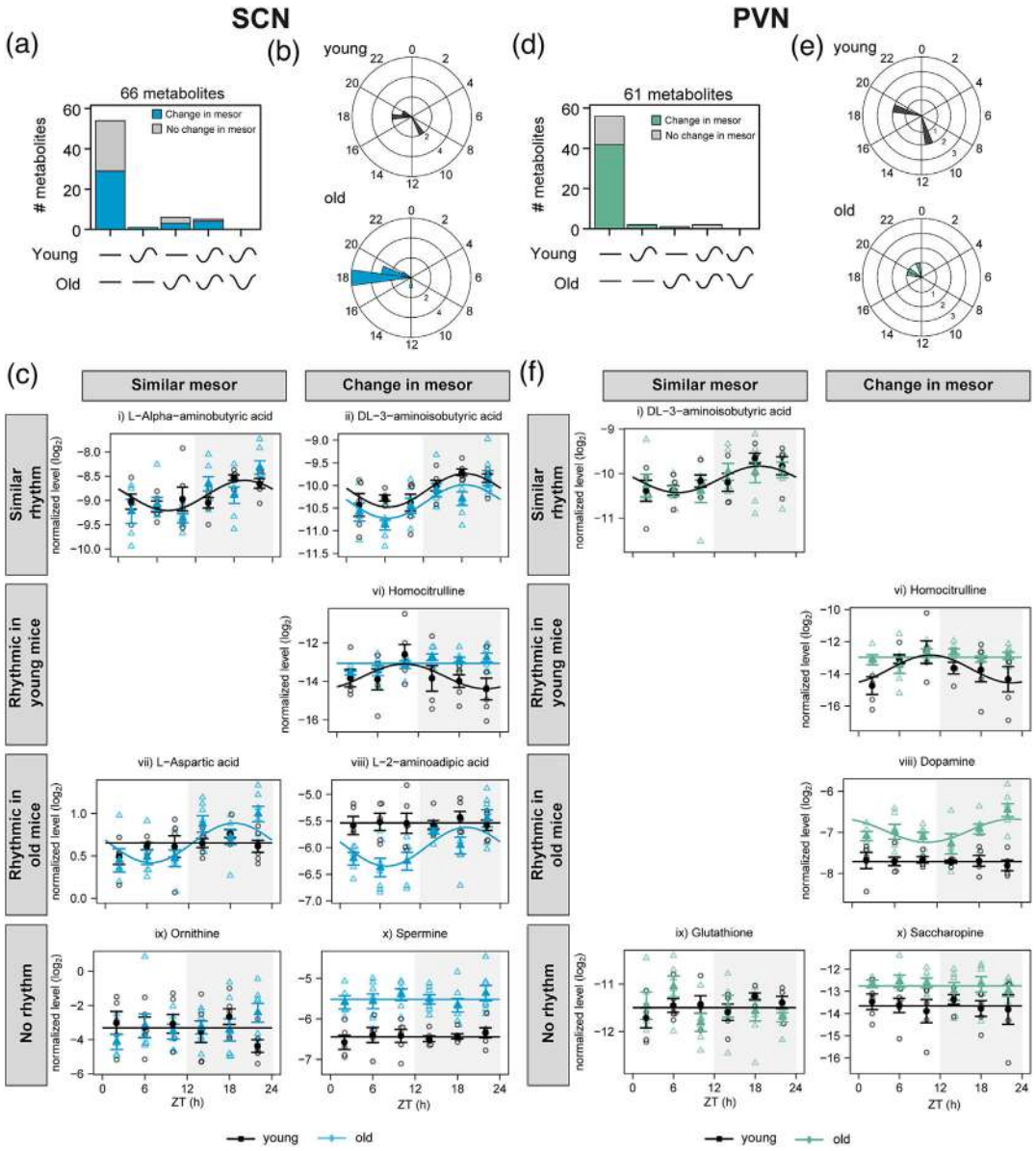

FIGURE 3 Metabolite rhythms and levels change in SCN and PVN in the course of ageing. (a,d) Categorisation of metabolites into categories based on differential rhythmicity analysis in SCN (a) and PVN (d). Bars show the number of metabolites per category: those that show a 24-h rhythm neither in the young nor in the old group; in the young group but not in the old group; in the old group but not in the young group; in both groups with similar amplitudes and peak times; or in both groups with different amplitudes and/or peak times. Differently coloured portions indicate the number of metabolites that have higher or lower levels in the old group compared to the young group. (b,e) Peak timing of rhythmic metabolites in the young (upper panel) and old (lower panel) group in SCN (b) and PVN (e). (c,f) Representative examples of metabolites assigned to one of the 10 different model categories the selected models, based on changes in average levels and rhythmicity shown for SCN (c) and PVN (f). Metabolite levels are represented in the $\log_2$ of the MS peak area divided by the internal standard. Data are shown as individual data points and as mean ± standard error of the mean. Lines depict model fits resulting from differential rhythmicity analysis. Grey areas mark the dark period (active phase), and light areas represent light period (resting phase). For SCN, no metabolites were assigned to model category iii, iv and v. For PVN, no metabolites were assigned to model category ii, iii, iv, v and vii.

of metabolites per model category for the SCN is given in Dataset S1, and a representative example for each category is shown in Figure 3c. In the PVN, 7% of the measured metabolites ($n = 4$ out of 61) were found to be rhythmic in young, and 5% ($n = 3$ out of 61) in aged mice, with two metabolites being rhythmic in both young and aged mice and two losing rhythmicity and one gaining rhythmicity in ageing (Figure 3d). Peak times were dispersed in both young and aged PVN tissue (Figure 3e). Furthermore, the abundance of 37 metabolites was

increased in the PVN of aged mice compared to young mice, while eight had a decreased abundance (Figure 3d). A list of metabolites per model category for the PVN is given in Dataset S1, and a representative example for each category is shown in Figure 3f.

3.2 | Tissue-specific alterations in polyamine levels and rhythmicity in ageing

Subsequently, we zoomed into the polyamine pathway, a metabolic pathway of special interest in the context of ageing, as it has been implicated in longevity and cardioprotection (Eisenberg et al., 2016) and has been linked to the circadian system, as discussed more extensively below (Zwighaft et al., 2015). We therefore explored the metabolites that are part of this pathway in more detail in our dataset (putrescine, spermidine and spermine; Figure 4a). While in plasma, levels of two out of three polyamines were significantly reduced in ageing, a significant increase was observed in ageing for all three polyamines in liver, PVN and SCN (except spermine in liver; Figure 4b), highlighting striking tissue specificity in the impact of ageing on the polyamine pathway. In plasma, 24-h rhythmicity of polyamines was observed neither in young nor in aged mice. In young mice, spermidine and spermine were rhythmic in liver, and putrescine was rhythmic in SCN and PVN. In aged mice, these rhythms were lost (except putrescine in SCN) (Figure 4b).

3.3 | Temporal coherence in the amine metabolome between tissues is lost in ageing

We next assessed the correlations of metabolites within and between the examined tissues as a marker of temporal coherence (Figure 5a). Correlations were considered of interest when the correlation coefficient r was ≤ -0.7 or ≥ 0.7. Between 6.0% and 13% of the correlations met this criterium in tissue of young mice. In ageing, the number of correlations increased in all tissues (Figure 5B), with 6.3%–18% meeting the criterium in aged mice. The largest increase was noticeable in the liver, where 207 combinations of metabolites correlated with $r \leq -0.7$ or $r \geq 0.7$ in young, versus 317 in aged liver tissue. A smaller number of correlations with $r \leq -0.7$, or $r \geq 0.7$ was observed between tissues (Figure 5c). In

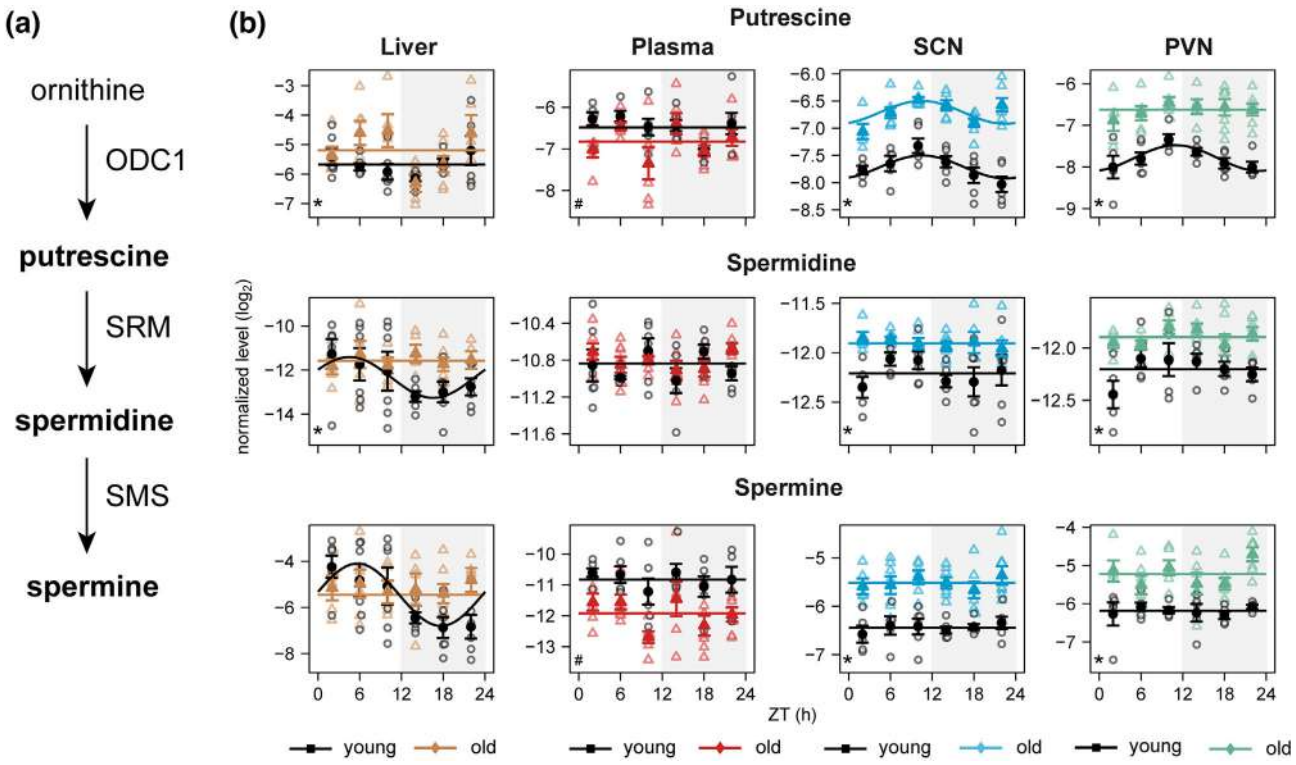

FIGURE 4 Changes in the polyamine pathway during ageing. (a) Overview of the polyamine pathway. Polyamine synthesis starts with the conversion of ornithine to putrescine by ornithine decarboxylase (ODC1). Putrescine is converted to spermidine by spermidine synthase (SRM), which is then converted by spermine synthase (SMS) to spermine. (b) 24-h profiles of polyamines (putrescine, spermidine, spermine) in liver, plasma, SCN and PVN. Data shown as in Figure 2c. Symbols: * indicates metabolite levels are significantly higher in old mice compared to young mice. # indicates metabolite levels are significantly lower in old mice compared to young mice.

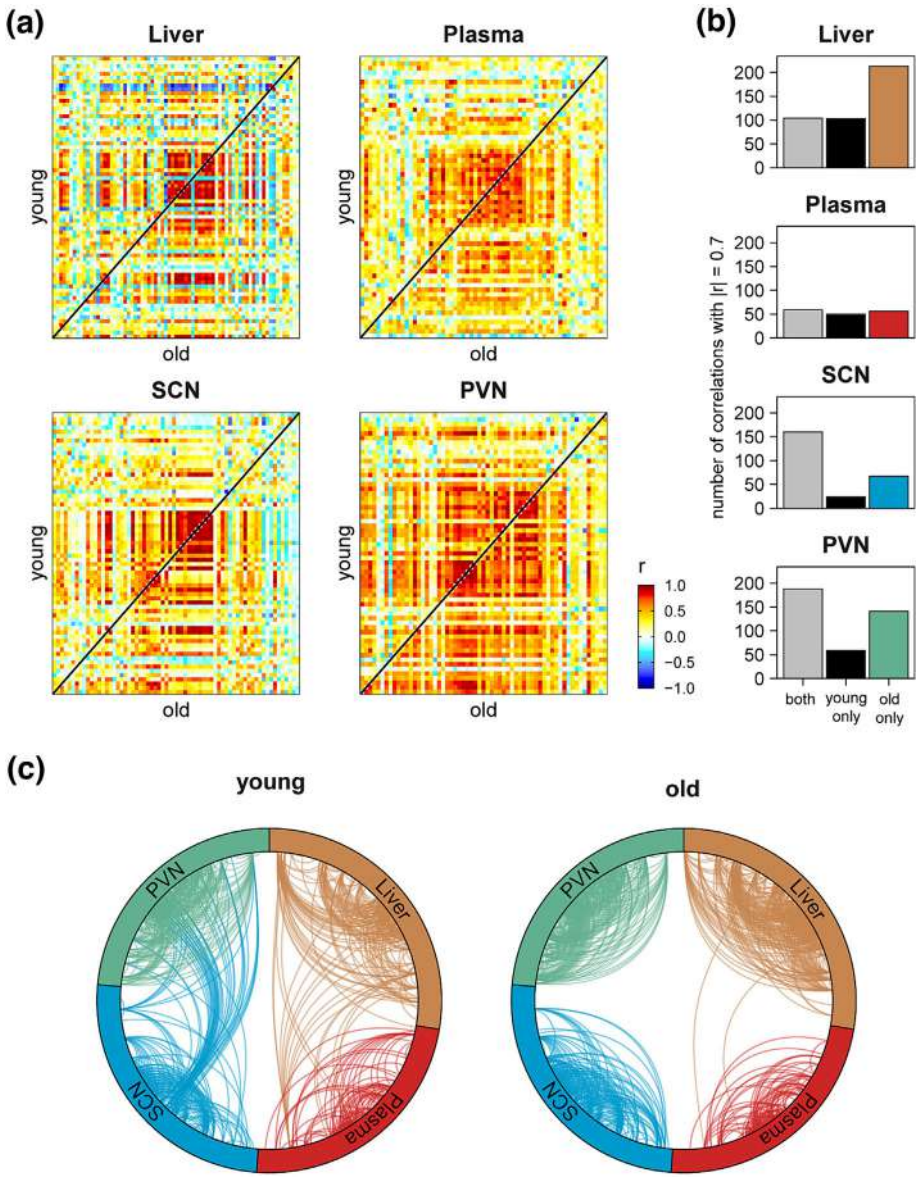

FIGURE 5 Within and between-tissue correlations of metabolite levels in young and old mice. (a) Matrices showing correlations between metabolites within each tissue. Colours indicate Pearson correlation coefficients (r). Top-left halves of the matrices show metabolite correlations in young mice; bottom-right halves show metabolite correlations in old mice. (b) Bar plots showing number of correlations between metabolite pairs with $-0.7 \leq r \geq 0.7$ in both young and old mice, in young mice only and in old mice only. (c) Circular plot showing within and between-tissue correlations. Lines connect metabolites with $r \leq -0.7$ or $r \geq 0.7$ within and between liver, plasma, SCN and PVN in young (left panel) and old mice (right panel).

young mice, inter-tissue correlations were exclusively found between SCN and PVN ($n = 48$) and between liver and plasma ($n = 17$) but not between these brain structures and peripheral tissues. In ageing, these inter-tissue correlations were almost entirely lost, with only two correlations meeting the threshold between liver and plasma.

4 | DISCUSSION

Given the interplay between the circadian clock, metabolism and ageing (Hood & Amir, 2017), a better understanding of the mechanisms underlying these relationships can contribute to the development of therapeutic targets to promote healthy ageing. Focusing on amine metabolism because of its relevance to ageing as well as circadian clock function (Acosta-Rodriguez et al., 2021; Zwighaft et al., 2015), we investigated the tissue-specific impact of ageing on 24-h metabolite rhythms using a targeted metabolomics approaches in SCN, PVN, liver and plasma. This resulted in a detailed description of ageing-related changes in the temporal coordination of amine metabolism across tissues. Overall, our data indicate that the abundance of many metabolites show a 24-h rhythm in young mice and that ageing severely affects these daily fluctuations, most notably in the liver. Moreover, in all tissues, many of the measured metabolites are found at significantly different average levels in tissue from aged compared to young mice. Finally, we found clear correlations between the metabolic profiles of liver and plasma and between SCN and PVN in young mice that were almost entirely lost in ageing. We conclude that ageing severely affects metabolite

levels, rhythmicity and systemic temporal coherence. This loss of coordination in ageing indicates disturbed communication between these tissues, which may have system-wide consequences.

We observed a significant ageing-associated reduction in the number of rhythmic amine metabolites in the liver but not in plasma, PVN and SCN, indicating that the impact of ageing is highly tissue specific. A potential explanation of the differences in the ageing-induced changes in metabolite profiles between SCN and liver tissue is the mounting evidence of resilience against disturbances including ageing (Fernandez et al., 2021), which seems to be founded in its highly organised and plastic neuronal network (Butler & Silver, 2009). In young mice, 51% and 36% of the detected metabolites showed a 24-h rhythm in liver and plasma, respectively, while this percentage was markedly lower in the SCN and PVN (9% and 7%, respectively). This difference between SCN and peripheral tissues is similar to the results from Dyar et al. (2018), showing rhythmicity in about 40% of the measured metabolites in liver and plasma and in about 12% of the metabolites in the SCN in control mice. It should be noted that the statistical analysis to determine 24-h rhythmicity differed between the two studies. Nevertheless, it is confirmative that direct visual and statistical comparison of the 24-h profiles of metabolites from the young mice in our dataset and the control mice in the study of Dyar et al. (2018) shows a high degree of similarity between the data, despite slight differences between the two study protocols (e.g. use of running wheels in our study, age of the mice, metabolomics platforms). This further supports the validity of our findings.

The observed decline in rhythmic metabolites in liver that occurs in ageing is reminiscent of the ageing-associated dampening of rhythms in the lipidome of adipose tissue that we observed in a previous study using tissue from the same animals as in the current study (Held et al., 2021). It has been shown previously that profound changes occur in the circadian organisation of liver transcriptome and specific metabolic processes during ageing (Sato et al., 2017). Furthermore, our study shows that this decline is highly tissue specific, as it was not observed in the other tissues. The preservation of 24-h rhythmicity in plasma metabolites parallels the finding that the prevalence of rhythmic lipids does not change with ageing in plasma samples collected from healthy young and middle-aged older humans (Rahman et al., 2023). Together, these results suggest that the plasma metabolome is minimally affected by ageing, which is relevant for the development of single-sample methods to determine circadian phase using metabolomics (Cogswell et al., 2021; Kasukawa et al., 2012; Woelders et al., 2023).

We found that for many metabolites, their average levels over the 24-h period were altered in the tissues of aged mice compared to young mice, with most metabolites displaying an ageing-associated increase in abundance in all tissues and fewer metabolites showing a decrease. In plasma, this change in abundance was more ambivalent: Levels of 17 metabolites were increased and 14 were decreased. This mixed picture is in line with observations in human plasma samples, in which ageing-related changes in amine metabolites have been observed, with varying direction, as recently reviewed (Panyard et al., 2022). The impact of ageing on the changes in the abundance of metabolites were more distinct in other tissues: in liver, for example, 48 metabolites (out of a total of 73) had increased levels, and only five were decreased, again highlighting that changes that occur in ageing are remarkably tissue specific. Such an overall increase in amine levels may be suggestive of an ageing-related increase in protein and amino acid catabolism (Lawton et al., 2008).

For an organism to function correctly, many processes within and between tissues need to proceed in a concerted manner. In our study, temporal coherence between metabolites within tissues seemed increased in ageing, while coherence between tissues is almost completely abolished, suggesting a profound reorganisation of the temporal coordination of amine metabolism in ageing. Specifically, in young mice, the correlation analysis between tissues reveals a strong association between the two brain areas, the SCN and PVN, as well as between the two non-brain tissues, the liver and plasma. It is somewhat surprising that the two brain areas and the non-brain tissues are so strictly separated, because all four tissues are physically and functionally strongly connected: the SCN innervates the PVN (Buijs et al., 1999; Kalsbeek & Buijs, 2002) and thereby regulates the rhythmic release of several hormones involved in feeding, behavioural activity and sleep (Buijs et al., 2019); the SCN is connected to the liver through the sympathetic and parasympathetic nervous system (Kalsbeek et al., 2004, 2010); and also the liver and plasma are evidently connected (Rui, 2014). However, it is clear that—at least in young mice—metabolite levels correlate most strongly between the tissues with the closest proximity to each other and these correlations are completely lost in ageing. Interestingly, exposing mice to a high-fat diet has a similar effect on the correlations of metabolite levels between different tissues (Dyar et al., 2018), implying that in both cases, homeostatic regulatory mechanisms are disturbed. In general, enforcing daily rhythms in feeding and fasting, activity and rest and timely exposure to light and darkness in ageing will likely re-enforce rhythms in metabolite profiles and be beneficial for the

coherence between tissues, thereby improving whole body homeostasis and health.

Zooming in on polyamines (i.e. putrescine, spermidine and spermine) because of their relevance in ageing (Hofer et al., 2022) as well as the circadian system (Zwighaft et al., 2015), we observed an ageing-related decrease in their plasma levels, in line with previous studies (Eisenberg et al., 2009; Nishimura et al., 2006; Zwighaft et al., 2015). However, this change was not mirrored in the other tissues, where the three polyamines were significantly increased with ageing. This tissue specificity requires further investigation, especially given the purported anti-ageing effects of spermidine supplementation (Eisenberg et al., 2016; Hofer et al., 2022; Madeo et al., 2018) as well as the observation that reduced levels of polyamines lead to a longer circadian period at the cellular and behavioural level (Zwighaft et al., 2015). Furthermore, our study supports the prior observation that polyamine synthesis in the liver is under circadian control (Atwood et al., 2011) and further shows that this circadian control is lost in ageing. Future studies are warranted to investigate whether the loss of 24-h rhythmicity in polyamine levels is merely associated with ageing or contributes to the development of ageing-related diseases.

Strengths of this study include the use of highly defined sample collection of the hypothalamic nuclei (exact volume and location) as well as improved sensitivity and chromatographic separation of amines owing to our derivatisation method compared to global untargeted methods. In addition, we used differential rhythmicity analysis, allowing us directly compare 24-h rhythms between two groups. Differential rhythmicity analysis has been shown to be superior to the commonly used separate analysis of two groups of rhythmic features that can lead to erroneous results (Kervezee et al., 2018; Pelikan et al., 2022). Several limitations should also be acknowledged. We performed our experiments in male mice, so to what extent our findings are generalisable to female mice should be a topic of future investigation, especially given the previously reported sex differences in the metabolome during ageing (Bresilla et al., 2022). In addition, given the potential influence of housing conditions, social hierarchy and microbiome composition on endocrine, immune and metabolic process (Lai et al., 2021; Lee et al., 2022; Wikoff et al., 2009), it remains to be investigated to what extent these factors affected our findings. Moreover, ageing is known to be accompanied by changes in circadian rhythms in activity and feeding (Buijink et al., 2020; Houtkooper et al., 2011). Since 24-h metabolite patterns readily adapt to reversed feeding rhythms (Damiola et al., 2000; Kervezee et al., 2019; Mauvoisin et al., 2014; Skene et al., 2018; Xin et al., 2021), altered feeding patterns could account for some of the effects of ageing observed in this study.

In conclusion, we provide a comprehensive overview of the ageing-associated changes in the temporal coordination of the metabolome across multiple tissues in male mice. Focusing on amino acids and biogenic amines because of their key role in ageing-related processes, we show that the abundance of a substantial set of metabolites show marked fluctuations across the 24-h period. The effect of ageing on these 24-h rhythms is highly tissue specific, with rhythmic metabolites in the liver being most severely impacted. In addition, in ageing, the temporal coherence among tissues is almost entirely lost, showing that the ageing process severely affects the homeostatic coordination of metabolic processes between tissues.

AUTHOR CONTRIBUTIONS

Conceptualisation: M. Renate Buijink, Stephan Michel and Thomas Hankemeier. *Investigation*: M. Renate Buijink, Michel van Weeghel, Amy Harms, and Devika S. Murli. *Formal analysis*: M. Renate Buijink and Laura Kervezee. Writing—original draft: M. Renate Buijink and Laura Kervezee. *Writing—reviewing and editing*: Stephan Michel and Laura Kervezee. *Visualisation*: M. Renate Buijink and Laura Kervezee. *Supervision*: Johanna H. Meijer, Thomas Hankemeier and Stephan Michel. *Funding acquisition*: Stephan Michel, Laura Kervezee and Johanna H. Meijer.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data and code that support the findings of this study are openly available Mendeley at https://doi.org/10.17632/57t9j8gm53.1.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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